Preparation and Analysis of Deuterium-Labeled Aspirin: Application to Pharmacokinetic Studies

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Abstract □ Inhibition of endogenous prostacyclin and thromboxane biosynthesis by aspirin is critically dose-dependent in humans. Gastrointestinal and hepatic hydrolysis may limit systemic availability of aspirin, especially in low doses, perhaps contributing to the biochemical selectivity of aspirin. Existing analytical methods do not permit determination of systemic bioavailability when low (<100 mg) doses of aspirin are administered. Deuterium-labeled aspirin (2-acetoxy[3,4,5,6-$^2H_4$]benzoic acid) was synthesized from salicylic acid by catalytic exchange and subsequent acetylation. Analysis of the compounds as benzyl esters by GC–MS followed extractive alkylation from plasma. Heptadeuterated compounds were used as internal standards. Simultaneous administration of tetradeuterated aspirin intravenously with native aspirin orally to anesthetized dogs permitted kinetic studies of both aspirin and salicylic acid. The sensitivity of the method is superior to published methods using HPLC and, thus, more applicable to studies of low dose aspirin. Pulse administration of stable isotope-labeled aspirin permits detailed and repeated studies of dose-related aspirin pharmacokinetics in humans.

Aspirin acetylates and irreversibly inhibits the enzyme cyclooxygenase[1] which catalyzes the transformation of arachidonic acid into thromboxane (TxA_2) and prostacyclin (PGI_2) via cyclic endoperoxide intermediates. TxA_2, the major product in the platelet, is a vasoconstrictor and potent stimulus to platelet aggregation, whereas PGI_2, the principal metabolite produced by endothelial cells, relaxes vascular smooth muscle and inhibits platelet function.[2,3] Interest in the disposition of extremely low doses of aspirin has been prompted by the suggestion that "selective" inhibition of TxA_2 formation by aspirin is more likely at doses <100 mg.[4,5] It is known that aspirin undergoes a substantial "first-pass" effect[6] which may substantially limit systemic bioavailability following oral administration of low doses. Aspirin is rapidly deesterified to salicylic acid in vivo,[7] and studies in vitro suggest that salicylic acid, under certain conditions, may competitively inhibit binding of aspirin to the cyclooxygenase enzyme.[8–10] Accumulation of salicylic acid occurs during chronic dosing with aspirin[11] and this may modify the pharmacodynamic effects of aspirin in vivo.

Prior studies of aspirin disposition have employed doses >300 mg[12–17] and have not been performed during continued drug administration. Previously described assay methods[18–21] lack the requisite sensitivity and specificity to characterize aspirin kinetics following doses <100 mg. In addition, the majority of studies have not employed parenteral administration of aspirin in the pharmacokinetic analysis. We have synthesized deuterium-labeled aspirin in quantities sufficient to perform pharmacokinetic studies in animals and humans, allowing coadministration of protonated and deuterated aspirin by different routes. In addition, we have developed a stable isotope dilution assay employing GC–MS which detects labeled and unlabeled aspirin and salicylic acid in plasma, following coadministration experiments. The method is based on principles previously reported by Rosenfeld et al.[22] The high sensitivity and specificity of this assay permits its application to pharmacokinetic studies when low doses of aspirin are administered. Additionally, the use of stable isotope labeling allows repeated kinetic studies during chronic aspirin administration without the risk of radiation exposure.

Experimental Section

Chemicals and Reagents—Salicylic acid (lot MC 101687, Aldrich Chemical Co., Milwaukee, WI), aspirin, *N*-bromosuccinimide, potassium fluoride, benzoyl peroxide, sodium sulfate, potassium dihydrogen phosphate, and disodium hydrogen phosphate were of analytical grade and used without further purification. Methanol, ethyl acetate, hexane, carbon tetrachloride, benzyl bromide, dichloromethane, ethanol, acetic acid anhydride, ether, glacial acetic acid, and hydrochloric acid were also of analytical grade and used without further purification. [$CH_2{}^2H$]Toluene, [$^2H_8$]toluene, [^2H]H_2O, and [^2H]NaOH were of the highest available purity (more than 99%) and obtained from Aldrich. Heparin (5000 IU/mL) and sodium bicarbonate 8.4% were purchased from the local hospital pharmacy as USP grade.

Synthesis of 2-Acetoxy[3,4,5,6-$^2H_4$]benzoic Acid—Salicylic acid (36.2 mmol) was dissolved in 50 mL of [^2H]H_2O by dropwise addition of 40% [^2H]NaOH in [^2H]H_2O. The solution was adjusted to 5% with regard to [^2H]NaOH after complete dissolution. NiAl alloy (0.5 g) was added slowly under vigorous stirring, and the solution was heated to boiling and refluxed for 48 h under continuous stirring. After cooling, the reaction was terminated by adding concentrated HCl (Fisher Scientific Co., Fair Lawn, NJ) until pH 2 was reached. The mixture was then extracted twice with ether (Burdick and Jackson, Muskegan, MI), dried over sodium sulfate (Sigma Chemicals, St. Louis, MO) and concentrated to dryness under a flow of nitrogen. The material was recrystallized from H_2O to give long white needles. The crystalline material was dissolved in 10 mL of acetic anhydride, 300 μL of H_2SO_4 (Burdick and Jackson) was added, and the solution was heated at 60°C for 15 min. After cooling to room temperature, 90 mL of H_2O was added and the product was allowed to crystallize. The material was removed by filtration and the collected precipitate was recrystallized twice from 30:70 EtOH:H_2O to give 14.1 mol (40% yield). This synthesis was previously described.[23] The deuterated salicylic acid was prepared by simple hydrolysis in 8.4% sodium bicarbonate for 48 h. The deuterated aspirin crystals were dissolved in absolute ethanol and passed through a 0.45-μm filter (Millipore Corp., Bedford, MA) under sterile conditions. Aliquots (50 mg) of the solution were placed into sterile vials. The vials were placed in a vacuum desiccator, dried, sealed, and kept at room temperature. A random sample of the batch was tested for pyrogens and sterility according to USP criteria.

Synthesis of α-Bromo-[$^2H_7$]toluene—*N*-Bromosuccinimide (1 mmol; Aldrich) was added to an equivalent amount of [$^2H_8$]toluene (Aldrich) and diluted with 2 mL of carbon tetra-

0022-3549/85/0200-0188$01.00/0

chloride (Burdick and Jackson). Benzoyl peroxide (3 mg; Aldrich) was added to initiate the reaction which was performed under reflux for 60 min. The solution was filtered and concentrated at room temperature under a gentle flow of nitrogen. The residue was used in the further preparation of the internal standards as described below.

Synthesis of [$^2H_7$]Benzyl 2-Acetoxybenzoate and [$^2H_7$]Benzyl 2-Hydroxybenzoate—Aspirin and salicylic acid (10 mg each) were added to 4 mL of a KH_2PO_3:Na_2HPO_3 buffer (Burdick and Jackson) adjusted to pH 6.5 and containing 0.05 M tetrahexylammonium hydroxide (ICN Pharmaceuticals, Plainview, NY). α-Bromo-[$^2H_7$]toluene (200 μL) and dichloromethane (4 mL; Burdick and Jackson) was added, and the sample was shaken by a mechanical shaker (Eherbach Corp., Ann Arbor, MI) for 10 min. The organic phase was evaporated, the residue was dissolved in hexane, and the solution was subjected to repeated chromatographic purification on silica gel SEP PAK columns (Waters Instruments, Milford, CT). The column was washed with 10 mL of hexane and the derivatives eluted with ether and stored at −20°C in ethyl acetate (Burdick and Jackson).

Liquid Chromatographic Analysis and Sterility Testing—One-tenth of the vials containing 50 mg of tetradeuterated aspirin were subjected to stability and sterility testing. The procedure for sterility testing was as follows. The 50-mg sample was dissolved in a 1.8-mL sterile solution of 8.4% sodium bicarbonate followed by further dilution with 8.2 mL of sterile water. This procedure ensured approximate isotonicity of the solution. Pyrogen and sterility tests were then performed according to USP, articles 26 and 34. The stability of the lyophilized, deuterated aspirin preparation was estimated by measurement of aspirin and salicylic acid by HPLC. HPLC was performed using a Waters model 6000 pump connected to a Rheodyne R Injection Port (Rheodyne Corp., Cotati, CA), a μ-Bondpack C_{18} column (Analytichem International, Harbor City, CA), and a variable-wavelength UV detector used at 280 nm (Spectromonitor III; LDC Instruments, Riviera Beach, FL). The solvent system employed was methanol:water:glacial acetic acid (40:60:1) (Burdick and Jackson).

Nuclear Magnetic Resonance and Gas Chromatographic–Mass Spectral Analysis—^1H NMR of the compounds was performed on a JEOL FX 90Q Ft. Instrument (JEOL Analytical Instruments Division, Cranford, NJ) in 50 mg/mL of $CDCl_3$ and compared with analytically pure aspirin and salicylic acid. The resonance spectrum showed disappearance of the triplets originating from the aromatic protons, indicating deuterium substitution. A signal with a shift relative to Me_4Si (Aldrich) of δ 7.2 ppm, however, indicated incomplete substitution of the proton located in the 6-position in the ring. The compounds themselves were analyzed by GC–MS using an LKB 9000 instrument (LKB Instruments, Gaithersburg, MD) with a direct probe inlet and were analyzed as their benzyl esters after GC separation on a 6 foot (1.8 m) 3% OV-17 column at 230°C interfaced with the same instrument. Mass spectra (Riber Mag 10-10; Nermag Corp., Houston, TX) of the tetradeuterated material at direct inlet confirmed the identity of the synthetic material. The molecular ion (M^+) at m/z 184 showed the presence of four deuterium atoms in the molecule, and initial mass fragmentographic analysis of the material demonstrated 66% as tetradeuteroaspirin, 32% as trideuteroaspirin, and <0.1% as the tetraprotonated compound. No evidence of other differences in the chemical structure of the compounds was found. Subsequent spectra of the benzyl esters of both aspirin and salicylic acid are shown in Fig. 1. Although fragmentation was not extensive, major ions at m/z 120 and 228 in the unlabeled species and at 124 and 232 in the labeled species indicate retention of the deuterium in the ring structure.

Analytical Procedure—The analytical procedure was as follows: 300 μL of plasma was mixed with 4 mL of a 0.06 M KH_2PO_3/Na_2HPO_4 buffer, pH 6.5, containing 0.05 M tetrahexylammonium hydroxide, 5 μL each of an internal standard solution, containing suitable amounts of aspirin and salicylic acid–heptadeuterobenzyl esters, 20 μL of benzyl bromide (Aldrich), and 4 mL of dichloromethane. The sample was extracted in a mechanical shaker for 15 min and centrifuged at 3000 rpm at 20°C. The upper layer and colloid interface were removed under reduced pressure and the organic phase was reduced to dryness under a constant stream of nitrogen. The sample was then partitioned between 2 mL of hexane and 2 mL of water by vortex mixing followed by centrifugation. The hexane phase

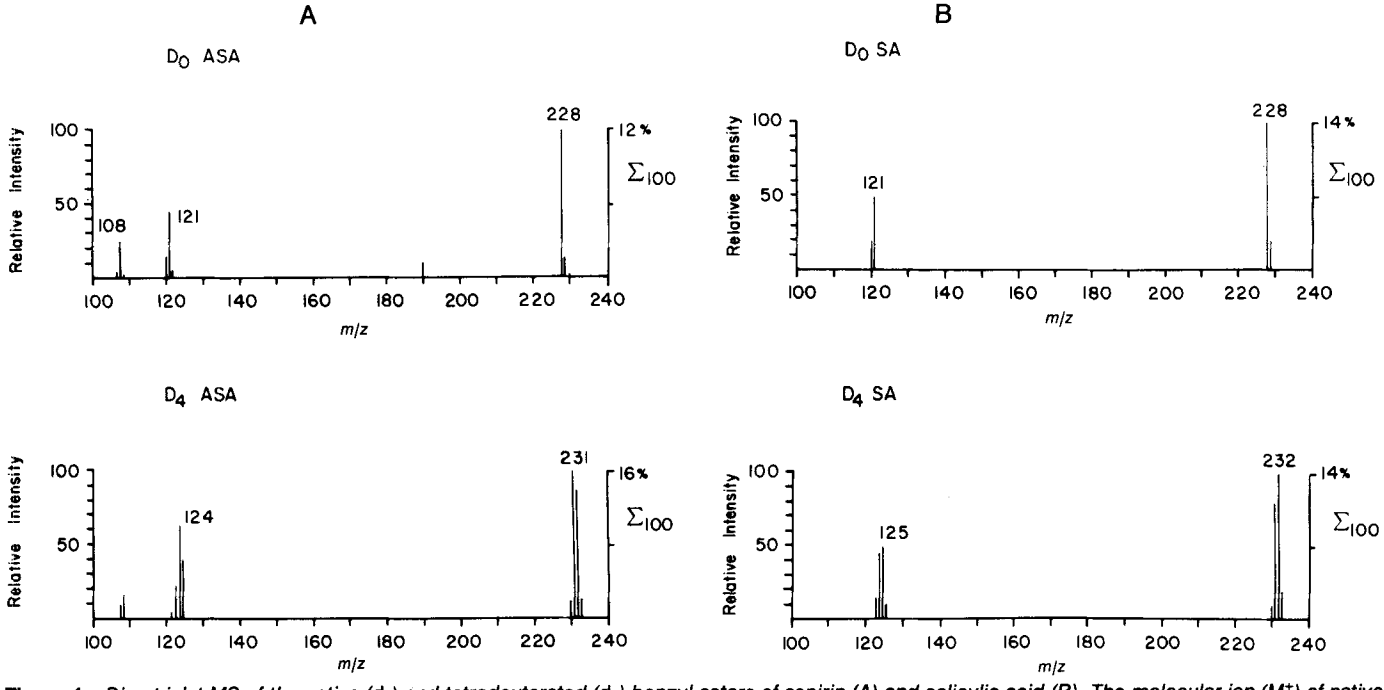

Figure 1—*Direct inlet MS of the native (d_0) and tetradeuterated (d_4) benzyl esters of aspirin (A) and salicylic acid (B). The molecular ion (M^+) of native aspirin is located at m/z 228; major ion peaks at m/z 232 and 231 indicate a mixture of d_4 and d_3 aspirin (A) and salicylic acid (B) in the lower panel.*

was then added to a test tube containing 0.5–1 g of sodium sulfate and shaken by hand for 30 s, decanted, and reduced to dryness under nitrogen. The residues were dissolved in 10–50 μL of ethyl acetate prior to analysis by an HP5710 GC interfaced with an HP5980A MS (Hewlett-Packard, Palo Alto, CA) using a 3 foot (0.9m) 3% SP-2250, column at 230°C. These conditions permitted selected-ion monitoring of the d_0, d_4, and d_7 materials at m/z 228, 232, and 235, respectively, at a conveniently short retention time.

Pharmacokinetic Experiments—To test this approach to the study of aspirin kinetics, we performed preliminary experiments in the dog. Unlabeled aspirin dissolved in equimolar amounts of 0.1 M sodium bicarbonate was administered intravenously or through a nasogastric tube to two conscious mongrel bitches (24 and 26 kg) coincident with intravenous injection of the tetradeuterated material. Peripheral venous blood samples were drawn for analysis prior to drug administration and at 3, 5, 10, 15, 30, 45, 60, 75, 90, 110, 180, and 240 min after drug administration. The blood samples (5 mL) were drawn into 100 μL of heparin and 100 μL of a 50% solution of potassium fluoride. The samples were stored on ice, immediately centrifuged at 3000 rpm for 15 min at 4°C, and the plasma was stored at −70°C prior to analysis of d_0- and d_4-aspirin. In a second experiment (dog A_2) a much higher (20 mg/kg) oral dose of d_0-aspirin was coadministered with the original intravenous dose (2 mg/kg) of d_4-aspirin.

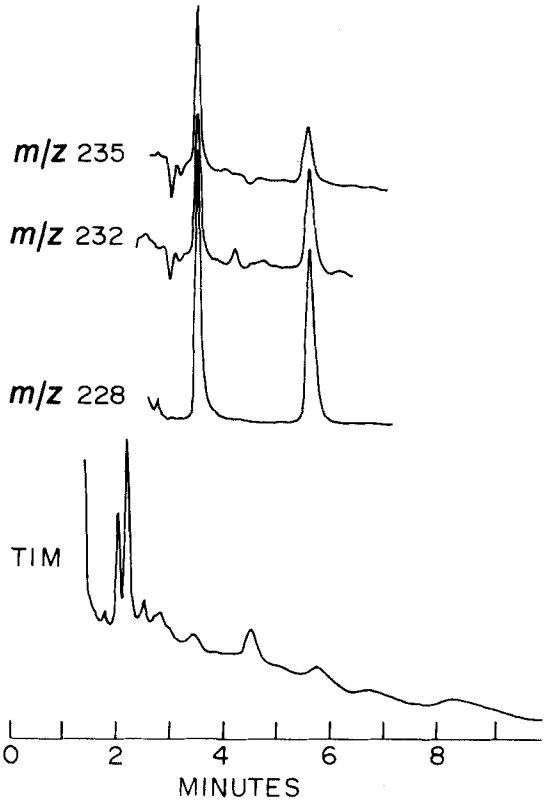

Figure 2—*Single ion traces (m/z 228, 232, and 235) and total ion current (TIM) of native, tetradeuterated, and heptadeuterated aspirin and salicylic acid (2 μg/mL in plasma).*

Table I—Ratio of Native (d_0) and Tetradeuterated (d_4) Aspirin (μg/mL) Following Oral Ingestion of a Capsule Containing d_0-Aspirin (40 mg)[a] and d_4-Aspirin (40 mg)[b]

Minutes, Post-dose	d_0-Aspirin	d_4-Aspirin	d_0/d_4
Predose	ND[c]	ND	ND
5	ND	ND	ND
10	0.24	0.18	1.3
15	0.51	0.53	0.96
20	0.64	0.58	1.1
30	0.61	0.53	1.2
45	0.33	0.38	0.85
60	0.18	0.17	1.06
90	0.09	0.1	0.9

[a] Ref. 17. [b] A fasting healthy male volunteer ingested a capsule containing a mixture of d_0- and d_4-aspirin. The approximate identity of the concentrations of the native and tetradeuterated species in plasma suggests that an isotope effect was unlikely. [c] ND indicates that the relevant compound was not detected.

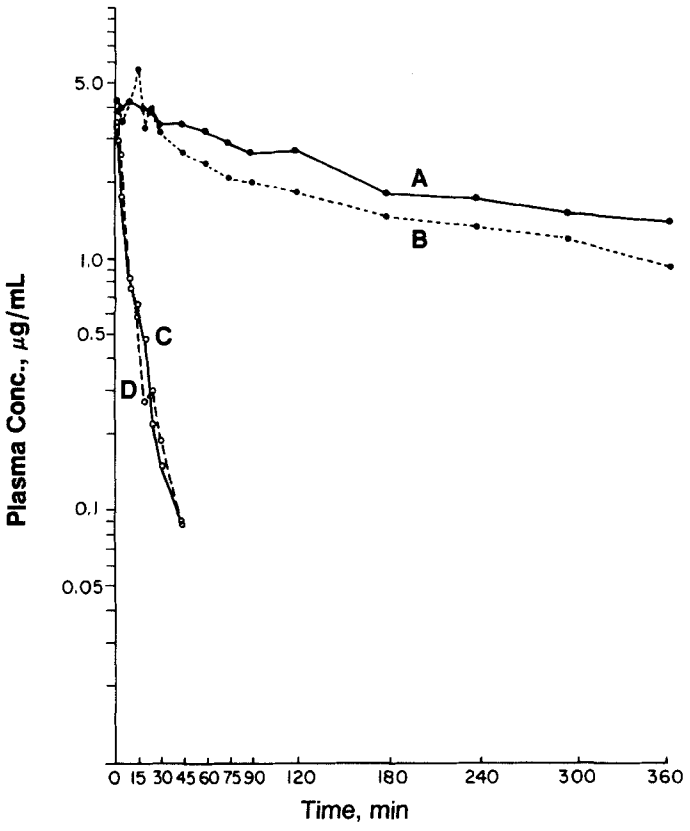

Figure 3—*Plasma concentrations of native (d_0) and tetradeuterated (d_4) aspirin and salicylate following coadministration of d_0-and d_4-aspirin intravenously (2 mg/kg) to a conscious mongrel bitch. Key: (A) d_4-salicylate; (B) d_0-salicylate; (C) d_0-aspirin, (D) d_4-aspirin.*

Results and Discussion

Preparation of 3,4,5,6-Tetradeuteroacetylsalicylic Acid—Catalytic exchange of the aromatic protons in salicylic acid was attempted with 0.1% palladium on carbon and 0.1% platinum oxide in ^2H_2O at temperatures ≤180°C. However, the exchange rate was slow and significant degradation of the salicylate was observed. An efficient reaction using Raney NiAl alloy under strongly alkaline conditions[23] was selected as this resulted in sufficient preservation of the starting material.

Validation of the Method—According to the tests performed, the pharmaceutical preparations of the tetradeuterated aspirin were pyrogen free and sterile. HPLC analysis confirmed >95% chemical stability and purity of the preparations after 8 weeks at room temperature in the dry vials. Furthermore, analysis of the isotonic bicarbonate solution of the drug, kept at 4°C for 2 h, showed <5% hydrolysis. We found no indication of in vitro hydrolysis of aspirin in the frozen plasma samples kept for up to 4 weeks. The overall hydrolysis of aspirin during the sampling and analytical procedure was <7%. Using the described procedure, the limit of sensitivity of the assay was approximately 10 ng/mL and the intra-assay coefficient of variation was <5% for both aspirin and salicylic acid. The

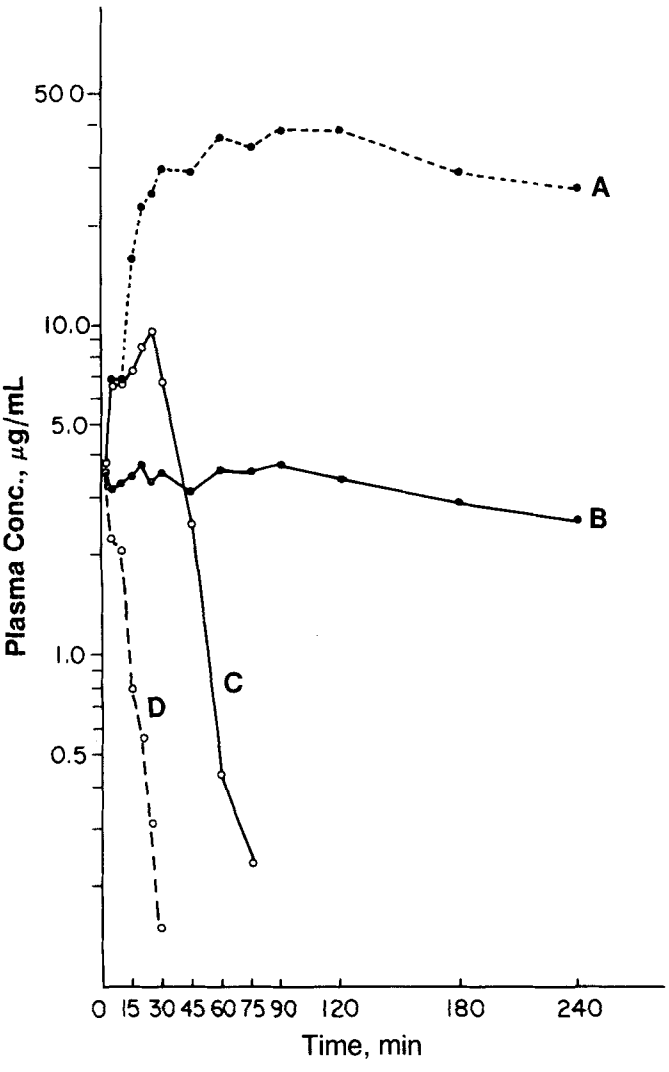

Figure 4—*Plasma concentrations of native (d_0) and tetradeuterated (d_4) aspirin and salicylate following coadministration of d_0-aspirin (20 ng/kg) orally and d_4 aspirin (2 mg/kg) intravenously to a conscious mongrel bitch. Key: (A) d_0-salicylate; (B) d_4-salicylate; (C) d_0-aspirin; (D) d_4-aspirin.*

recovery of aspirin and salicylic acid from comparable plasma and aqueous samples was quantitative. These results thus confirm the use of extractive alkylation for reliable determination of aspirin and salicylic acid.[22]

Figure 2 shows the total ion current as well as single ion traces for the benzyl-d_0-aspirin (m/z 228), the benzyl-d_4-aspirin (m/z 232), and the benzyl-d_7-aspirin (m/z 235) and salicylic acid in concentrations of 2 μg/mL added to plasma in vitro. The ions monitored are molecular ions for the salicylate–benzyl ester and MS analysis of the GC peak confirmed the results previously reported.[22] The first peak with a retention time of 3.5 min was identical to salicylate–benzyl ester whereas the second peak, retention time 5.7 min, showed the same base ion but with a minor M^+ of +42, corresponding to an acetyl group. The aspirin–benzyl ester was completely stable on the GC, thus separating it from salicylic acid. Concentrations of aspirin and salicylic acid were computed from peak height ratios of both the native and tetradeuterated species to the heptadeuterated internal standard. Recovery of the internal standards through the extraction and derivatization procedures exceeded 95% for both aspirin and salicylic acid, and the reproducibility of this recovery was satisfactory (coefficients of variation <6%; n = 10). Calibration curves of the standards were linear up to at least 100 μg/mL of aspirin and salicylic acid in plasma (regression coefficients >0.95). At the higher concentrations of the standard, sample dilution was performed to avoid the possibility of overloading the GC–MS.

The convenience of monitoring only one ion for each pair of compounds greatly reduces the time required for analysis, since all six compounds can be determined from one sample. The selectivity of the method is apparent from the difference between the single ion traces and the total ion trace. Before analysis, the ion peak shape was routinely adjusted to a suitable resolution power and, using the quadropole instrument, it was possible to maintain a high sensitivity. Blank values in plasma were less than 10 ng/mL and samples spiked with the isotopes alone showed less than 0.1% signal in the other ion channels.

Pharmacokinetic Experiments—Simultaneous administration of unlabeled and labeled drug has been utilized to estimate systemic bioavailability in vivo.[24] The use of stable isotope labeling avoids the risk of radiation hazard, a feature of particular convenience where repeated studies are desired. The time–concentration curves from the dog experiments are shown in Figs. 3 and 4. In dog A_1 the approximate identity of disposition of the labeled and unlabeled material (Fig. 3) indicated the absence of a kinetic isotope effect.[24] Absorption of orally administered drug by dog A_2 was slow and peak concentrations of d_0-aspirin were evident in venous plasma 30 min after dosing (Fig. 4). Despite the dose-related differences in plasma concentrations, the elimination half-lives of d_0- and d_4-aspirin (15.1 and 16.2 min) were similar. To address the issue of an isotope effect more formally, an oral dosing experiment was performed to maximize the likelihood of such an event. Native aspirin (Merck, Sharpe and Dohme Laboratories, Rahway, NY) and tetradeuterated aspirin, 40 mg each, were mixed together in a single capsule and administered orally to a healthy volunteer; the plasma concentrations of aspirin were measured after dosing. Once again, the approximate identity of disposition of the native and tetradeuterated material suggested that an isotope effect was unlikely (Table I).

In conclusion, we have synthesized sufficient tetradeuterated aspirin to permit pharmacokinetic studies in animals and humans. In addition, we have utilized a GC–MS assay employing a heptadeuterated internal standard in preliminary experiments which demonstrate the feasibility of measuring systemic bioavailability of extremely low doses of aspirin with this technique. If low dose aspirin is subject to a major degree of first-pass hepatic metabolism in humans, exposure of platelet cyclooxygenase might occur predominantly in the presystemic circulation, whereas the amounts of aspirin escaping hepatic hydrolysis might be insufficient to inhibit PGI_2 biosynthesis by the systemic vasculature. Investigations integrating the pharmacokinetics with the observed pharmacodynamics of low aspirin doses are thus desirable. Furthermore, the use of deuterium labeling permits repeated studies of aspirin pharmacokinetics and possible salicylic acid–aspirin interactions during the accumulation of salicylic acid which occurs in plasma during chronic administration of aspirin.[25,26]

References and Notes

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Acknowledgments

We wish to thank Drs. D. Taber and J. Clanton for their advice and the preparation and sterility testing of tetradeuterated aspirin.

Supported by grants HL 30400, GM 15431, and RR 05424 from the U.S. Public Health Service. Dr. Pedersen in the recipient of a Fellowship from the Danish Medical Research Council. Dr. FitzGerald is the recipient of a Faculty Development Award from the Pharmaceutical Manufacturer's Association Foundation.