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GAS CHROMATOGRAPHIC SEPARATION OF OPTICALLY ACTIVE ANTI-INFLAMMATORY 2-ARYLPROPIONIC ACIDS USING (+)- OR (−)-AMPHETAMINE AS DERIVATIZING REAGENT

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SUMMARY

A method is described for the derivatization of several non-steroidal anti-inflammatory arylalkanoic acids (ibuprofen, ketoprofen, naproxen, fenoprofen, flurbiprofen, pirprofen, cicloprofen, tiaprofenic acid, etodolic acid) with optically active amphetamine. The usefulness of this reagent compared to α-methylbenzylamine is described. The enantiomers are separated as diastereoisomers using capillary gas chromatography with nitrogen—phosphorus detection. The procedure is readily applied to the quantification of the enantiomers in urine and plasma samples.

INTRODUCTION

The interaction between a chiral drug and its receptor is inherently diastereomeric owing to the asymmetric character of the biological system [1, 2]. Such chiral agents may exhibit a significant degree of stereoselectivity in their actions and, in fact, only one isomer (eutomer) may be active; the other (distomer) is often much less active or, in some cases, inactive. The activity ratio of eutomer to distomer is described as the eudismic ratio and is a measure of stereoselectivity [3].

The stereoselective disposition (absorption, distribution, excretion) of chiral 2-arylpropionic acid non-steroidal anti-inflammatory drugs (2-APA-NSAIDs, Fig. 1) has recently attracted significant attention [4]. Of particular interest is the unidirectional bioconversion of the distomer (*R*-configuration, generally levorotatory) to the eutomer (*S*-configuration, dextrorotatory) [5,6]. It becomes apparent that a derivatization procedure for the resolution and measurement of the individual enantiomers present in biological fluids is essen-

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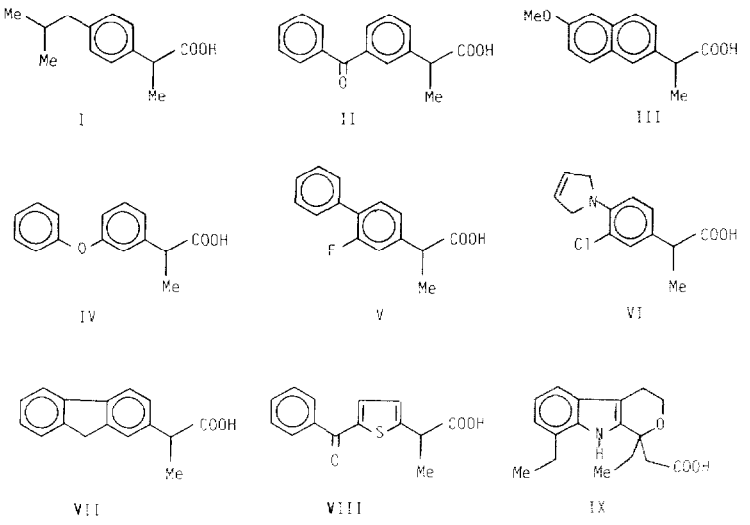

Fig. 1. Structures of the arylalkanoic non-steroidal anti-inflammatory drugs ibuprofen (I), ketoprofen (II), naproxen (III), fenoprofen (IV), flurbiprofen (V), pirprofen (VI), ciclo-profen (VII), tiaprofenic acid (VIII) and etodolic acid (IX).

tial for the proper interpretation of pharmacokinetic data obtained following the administration of drugs as racemates.

The resolution of enantiomers is most frequently achieved by either of two methods. Direct separation on chiral high-performance liquid chromatographic (HPLC) or gas chromatographic (GC) columns, or by diastereoisomer formation (by reaction with an optically pure resolving agent) followed by chromatography on an optically inactive column. The latter method is often preferred when the drug to be measured is present in very low concentrations in biological samples. Detection and quantification may then be improved by selecting a chiral resolving agent which will confer enhanced detector sensitivity to the resulting diastereoisomers. In this report, we describe a GC method for the efficient separation of enantiomeric pairs of 2-APA-NSAIDs as diastereoisomeric α-methylbenzeneethanamides.

The chromatographic methods for the determination of the enantiomeric composition of anti-inflammatory 2-arylpropionates have been reviewed [5]. Most studies involve formation of diastereoisomeric amides using optically active α-methylbenzylamine (MBA) followed by GC or HPLC. We prefer GC because combination with mass spectrometry (MS) allows for confirmation of structure. This capability is particularly important for metabolic studies where metabolite structures are unknown. Whereas (+)- and (−)-MBA are versatile derivatizing reagents, we have found that optically active α-methylbenzeneethanamine (amphetamine, AM) is an attractive alternative.

EXPERIMENTAL

Chemicals and reagents

All solvents were ACS grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.) and glass-distilled prior to use. Water was distilled and deionized (Milli-Q reagent

water system, Millipore, Bedford, MA, U.S.A.). The following NSAIDs were obtained as gifts: ketoprofen and (+)-ketoprofen (Rhone-Poulenc, France); ibuprofen (Upjohn, Don Mills, Canada); naproxen (Syntex, Palo Alto, CA, U.S.A.); fenoprofen (Eli Lilly, Indianapolis, IN, U.S.A.); flurbiprofen (Boots, Nottingham, U.K.); pirprofen (Ciba-Geigy, Basle, Switzerland); cicloprofen (E.R. Squibb, Princeton, NJ, U.S.A.); tiaprofenic acid (Roussel, Montreal, Canada); racemic, (+)- and (−)-etodolic acid (Ayerst, New York, NY, U.S.A.). N-Trifluoroacetyl-L-prolyl chloride (TPC) was obtained from Regis (Morton Grove, IL, U.S.A.) while 1,1'-carbonyldiimidazole (CDI), *S*-(−)- and *R*-(+)-MBA were purchased from Aldrich (Milwaukee, WI, U.S.A.). *S*-(+)- and *R*-(−)-AM sulfate were gifts of Health and Welfare (Canada) although they are also available commercially (Sigma, St. Louis, MO, U.S.A.).

Apparatus

The gas chromatograph was a Hewlett-Packard 5730A equipped with a nitrogen–phosphorus detector, a 18740B capillary column controller and a 3390A integrator–recorder (Hewlett-Packard, Palo Alto, CA, U.S.A.). Mass spectra were obtained on a VG 7070E gas chromatograph–mass spectrometer–data system (Analytech Instrumentation and Service, St. Laurent, Canada). The Reacti-Therm system was purchased from Pierce (Rockford, IL, U.S.A.) and the Savant Speed Vac concentrator–evaporator 100H from Emerston Instruments (Scarborough, Canada). All solvent evaporation procedures utilized this apparatus.

Chromatographic conditions

The fused-silica capillary column (12 m × 0.2 mm I.D.) was coated (film thickness 0.33 μm) with a high-performance cross-linked methyl silicone film (Hewlett-Packard). The operating conditions were: injector, 250°C; detector, 300°C; column, 100–270°C (32°C/min) except for ibuprofen, 100–220°C (16°C/min). The column conditions for determining the enantiomeric purity of *S*-(+)- and *R*-(−)-AM were 100–160°C (16°C/min). The gas flow-rates were: helium (carrier), 2 ml/min; hydrogen, 3 ml/min; air, 50 ml/min. The column head pressure was maintained at 0.85 bar.

Determination of the enantiomeric purity of S-(+)- and R-(−)-amphetamine

The optical purity of each derivatizing reagent was determined by reaction with TPC [7]. The commercially available TPC reagent was found to contain a small amount of the D-isomer presumably as a result of epimerization during synthesis and/or storage [8,9]. For these studies we therefore utilized TPC freshly prepared according to a published procedure [10]. It has been reported that a methylene chloride solution of TPC is stable for four months at −20°C [11].

A toluene solution containing optically active AM free base (10 μg) was evaporated to dryness and the residue reconstituted with chloroform (1 ml). TPC was then added (30 μl of a freshly prepared 0.1 *M* methylene chloride solution), followed 15 min later by triethylamine (50 μl). The reaction mixture was then mechanically shaken for 15 min and washed sequentially with 5 *M* hydrochloric acid (1 ml) and water (1 ml). The organic layer was dried over

magnesium sulfate, evaporated, and the residue reconstituted with toluene (100 μl). A 1-μl aliquot was then analyzed by GC as described above. Under these conditions, the retention times of *R*-(−)-AM-TPC and *S*-(+)-AM-TPC were 12.19 and 12.71 min, respectively. The optical purity of *S*-(+)- and *R*-(−)-AM exceeded 97%.

Preparation of test solutions

Standard stock solutions of the NSAID (1 mg/ml) were prepared in methanol and stored at 4°C. Test solutions (100 μg/ml) were prepared from stock prior to derivatization. Standard solutions of CDI (100 μg/ml) were prepared fresh daily in chloroform or methylene chloride while solutions of optically active AM sulfate (equivalent to 500 μg of free base per ml) were prepared in water and stored at 4°C.

Derivatization of test solutions

Test solutions (100 μl) containing the NSAID were added to phosphate buffer, pH 7 (1 ml) acidified to pH 1 with 5 *M* hydrochloric acid (100 μl) and extracted with toluene (4 ml). A 100-μl portion of *S*-(+)- or *R*-(−)-AM solution was basified with 1 *M* sodium hydroxide (100 μl) and extracted with toluene (4 ml). The two extracts were combined in 100 × 13 mm glass tubes and evaporated to dryness. The resulting residue was reconstituted with 1 ml of chloroform or methylene chloride containing 100 μl of CDI stock solution. The tubes were screw-capped and sealed under a nitrogen atmosphere and heated for 2 h at 85°C in the Reacti-Therm system. After cooling to room temperature, the reaction mixture was washed with 1 *M* hydrochloric acid (1 ml), the organic phase dried over magnesium sulfate and evaporated to dryness. The residue was reconstituted with toluene (100 μl), and 1 μl of this solution analyzed by GC.

Derivatization of biological samples

Urine and plasma samples (1 ml) were diluted with phosphate buffer, pH 7 (1 ml) and to the mixture were added various quantities of ibuprofen (0.25–10 μg) and *p*-methoxyphenylacetic acid (2.5 μg) as internal standard. After thorough mixing, 1 *M* sodium hydroxide (100 μl) was added to hydrolyze ester conjugates (urine only) and to permit the removal of basic impurities when the sample is washed with diethyl ether (1 ml). The washed sample was then acidified to pH 1 using 1 *M* hydrochloric acid (100 μl). The free acid (ibuprofen) was extracted into toluene (4 ml) and this extract combined with a toluene solution (4 ml) of optically active AM. The derivatization procedure was continued as described above.

RESULTS AND DISCUSSION

The quantification of 2-APA enantiomers in biological media has been reported using HPLC, GC, thin-layer chromatography and radiochemical methods [5]. Of these, HPLC and GC appear to be the most frequently applied techniques; with a few exceptions [12,13], optically active MBA has been the most popular derivatizing reagent for both chromatographic procedures. Thus,

diastereoisomeric NSAID-α-methylbenzylamides are prepared directly by means of a coupling reagent such as CDI, or via the NSAID acid chloride prepared with thionyl chloride. We chose the CDI route because we found thionyl chloride to be ineffective for the preparation of diastereoisomeric tiaprofenic acid-benzeneethanamides. In addition, we required a general method equally applicable to the analysis of metabolites. It has been reported [14] that the hydroxylated metabolites of ibuprofen are readily dehydrated and hydrohalogenated with thionyl chloride.

There are comparatively few reports describing GC methods for the separation of NSAID enantiomers present in biological media. Racemic ibuprofen [14—16] and benoxaprofen [17] have been resolved as their MBA derivatives using GC with flame-ionization detection. When MBA is used as a derivatizing reagent, a large excess (6.5 mg) of CDI is required for the analysis of ibuprofen (0—50 μg) [15]. It has recently been reported [18] that increasing the proportion of CDI results in a corresponding increase in derivative yield. In that study, 52 mg of CDI were used for the derivatization of several NSAIDs (10—140 μg) with MBA. Formation of the symmetrically disubstituted α-methylbenzyl derivatives of urea, by reaction of MBA with the excess CDI, is minimized by

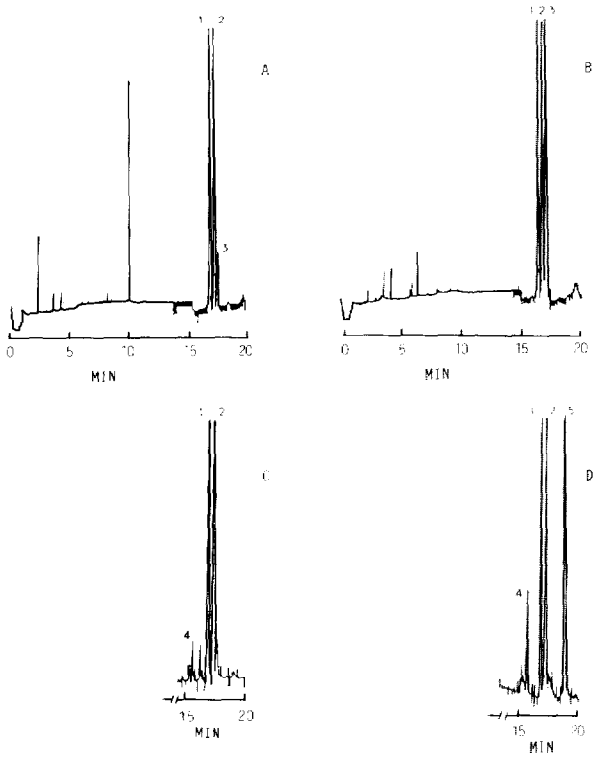

Fig. 2. Gas chromatographic resolution of (±)-ibuprofen (10 μg) with *S*-(+)-AM (50 μg): (A) using 10 μg CDI in a test sample; (B) using 50 μg CDI in a test sample; (C) urine sample; (D) plasma sample. Retention times are shown in Table I and chromatographic conditions are reported in the text. Peaks: 1 = *S*-ibuprofen,*S*-AM; 2 = *R*-ibuprofen,*S*-AM; 3 = unidentified component, also present in reagent blank; 4 = internal standard; 5 = endogenous plasma component.

the addition of acetic acid [15,18]. Dicyclohexylcarbodiimide (DCC)-mediated couplings in polypeptide syntheses were found to be essentially quantitative when equimolar amounts of DCC were used [19]. On this basis, it was felt that reduced quantities of chemically related CDI could be used in our procedure. Thus, our method uses only 10 μg of CDI for 10 μg of NSAID and therefore CDI is not present in large excess; the resulting chromatograms are virtually free of any interfering peaks (Fig. 2). Calibration graphs (Fig. 3) for ibuprofen, using this method, were linear over the concentration range 0.25– 10 μg/ml of plasma or urine. The minimum quantifiable concentration was 75 ng/ml of biological sample, with an on-column detection limit of 0.75 ng. When the quantity of CDI was increased to 50 μg, a significant amount of an unidentified component was obtained (Fig. 2B). This substance apparently forms via reaction between AM and CDI as it also appears in the reagent blank sample where an NSAID is not present. According to mass spectral evidence, it is not the α-methylphenylethyl derivative of urea. For the purposes of comparison, when we derivatized ibuprofen with MBA, using the recommended [15] quantities of CDI, numerous prominent peaks appeared in the chromatographic traces, particularly in the region prior to elution of the diastereoisomers (Fig. 4). These peaks were observed, in part, as a result of the greater resolving capability of capillary compared to packed columns. In addition, because of our column operating conditions, we detect several volatile components which may normally co-elute with the solvent when higher oven temperatures are used. It was felt that under our chromatographic conditions, MBA produced too many potentially interfering peaks in an area of the GC trace where metabolites might occur and, in addition, reduce the lifetime of the column. When the quantity of CDI was reduced to 10 μg, no reaction was observed between MBA and the NSAID.

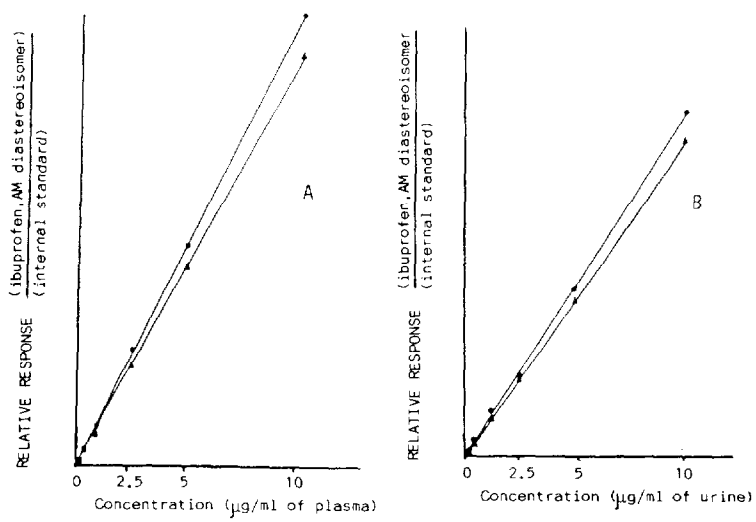

Fig. 3. Calibration graphs for ibuprofen,*S*-(+)-AM diastereoisomers over the concentration range 0.25—10 μg/ml in (A) plasma and (B) urine. (●) = *S*-(+)-ibuprofen,*S*-(+)-AM; (▲) = *R*-(−)-ibuprofen,*S*-(+)-AM. Gas chromatographic conditions are given in the text.

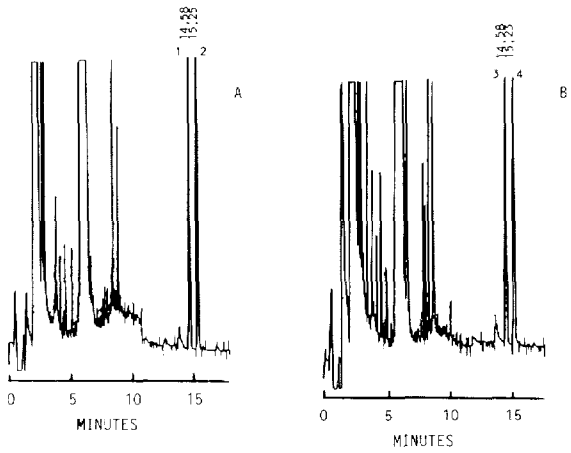

Fig. 4. Gas chromatographic resolution of (+)-ibuprofen (10 μg) with (A) *S*-(−)-MBA (25 μl) and (B) *R*-(+)-MBA (25 μl). In both cases, CDI (6.5 mg) was used as condensing agent (method of Vangiessen and Kaiser [15]). Retention times are shown in min. Peaks: 1 = *S*-ibuprofen,*S*-MBA; 2 = *R*-ibuprofen,*S*-MBA; 3 = *R*-ibuprofen,*R*-MBA; 4 = *S*-ibuprofen,*R*-MBA.

A further comparison of MBA with AM revealed that AM consistently gave a chromatographic peak-area ratio (i.e. ratio of peak areas of the two diastereoisomers) of 0.90—1.0 for all the NSAID diastereoisomers in this study. MBA gave equally good results with the two NSAIDs, fenoprofen and ibuprofen, selected for comparison. A ratio value of 1.0 is, of course, ideal in the analysis of racemates and suggests that the derivatizing reagent reacts equally well with both enantiomers. When the value deviates from unity, the calculation of enantiomer ratios in test samples requires the incorporation of a predetermined correction factor.

AM and MBA were essentially comparable in providing baseline resolution of the diastereoisomers, although peak separation was slightly greater with MBA. The retention times are also comparable; for example, ibuprofen-MBA diastereoisomers elute in approximately 15 min and the corresponding AM derivatives in 17 min using the same chromatographic conditions.

MBA is commercially available and conveniently handled as a liquid. We regenerate AM free base from the sulfate salt thereby introducing an additional extraction step. However, the sulfate salts are stable and easily recrystallized if desired. We have observed that during storage at 4°C, a colorless deposit forms in the neck of the bottle containing MBA. We have not determined the nature of this material nor is it clear whether it is a reflection of the stability of MBA.

The identity of the chromatographic peaks listed in Table I is based on the following information. (a) Optically pure *S*-(+)-ketoprofen was added to racemic ketoprofen and the mixture derivatized with *S*-(+)-AM. The area of the peak with retention time (t_R) of 17.11 min was augmented, indicating that the first-eluting stereoisomer has the *S,S* configuration. When *R*-(−)-AM was the resolving agent, the second-eluting peak was augmented, and therefore this diastereoisomer has the *S,R* configuration. (b) Naproxen is marketed as the

TABLE I

GC RETENTION TIMES (min) OF NSAID DIASTEREOISOMERS USING *S*-(+)-AMPHETAMINE OR *R*-(−)-AMPHETAMINE AS RESOLVING AGENTS

Fused-silica, methyl silicone capillary column (12 × 0.2 mm I.D.); oven temperature, 100–270°C (32°C/min) except for ibuprofen, 100—220°C (16°C/min); nitrogen–phosphorus detector; carrier gas (helium) flow-rate, 2 ml/min.

NSAID	*S*-(+)-Amphetamine		*R*-(−)-Amphetamine	
	*S,S**	*R,S**	*R,R**	*S,R**
Ibuprofen	17.11	17.46	17.10	17.46
Ketoprofen	12.80	13.40	12.84	13.47
Naproxen	12.57	12.89	12.62	12.80
Fenoprofen	19.18	19.31	19.13	19.46
Flurbiprofen	13.14	13.57	13.15	13.51
Pirprofen	9.73	10.01	9.74	10.01
Cicloprofen	11.69	12.05	11.72	12.06
Tiaprofenic acid	See text			
Etodolic acid	See text			

*Configuration of NSAID-amphetamine diastereoisomer.

S-(+)-enantiomer. When derivatized with *S*-(+)-AM, a sample of naproxen gave two peaks (96:4 ratio), the major, and first-eluting, peak being the *S,S* diastereoisomer and the minor component, presumably the *R,S* isomer. The order of elution reversed when *R*-(−)-AM was used as derivatizing agent. This information, along with similar results following HPLC analysis, suggests that the minor component does, in fact, arise from the presence of a small amount of *R*-(−)-naproxen. (c) It has been shown that following the administration of racemic ibuprofen to healthy volunteers, the *S*-(+)-isomer predominates in both plasma [12,15] and urine [15]. Following similar studies in our laboratories, the predominant isomer was found to elute first when derivatized with *S*-(+)-AM and therefore this diastereoisomer was assigned the *S,S* configuration. (d) It has been suggested [20,21] that for closely related compounds the relationship between the order of elution and configuration is generally consistent. Thus, with several structurally similar diastereoisomeric amides synthesized from *R*-(+)-MBA, the *S*-acid,*R*-amine diastereoisomers eluted (HPLC) prior to the *R*-acid,*R*-amine isomers [22]. The same elution order (HPLC) was observed in resolving racemic benoxaprofen with *R*-(+)-MBA [23]. When the diastereoisomers are separated by GC methods, however, the elution order is reversed compared to HPLC, i.e. the *S*-acid,*R*-amine diastereoisomers of simple amides [22], as well as ibuprofen [16], are retained longer. It has been reported [21,24] that the order of elution of enantiomers reverses upon reversal of GC column phase chirality. It is anticipated that reversing the chirality of the resolving agent should similarly reverse the elution order of the corresponding diastereoisomers. Thus, when *S*-(−)-MBA was used to derivatize benoxaprofen [17] and ibuprofen [14,15], the first-eluted GC peak corresponds to the *S,S* isomer. As expected, when analyzed by HPLC, the more polar *S,S*-isomers are retained longer on the column [13,18,23,25].

The elution orders reported in Table I are consistent with the preceding dis-

cussion; with *S*-(+)-AM as resolving agent, the *S*-acid,*S*-AM diastereoisomer elutes first while with *R*-(−)-AM, the *S*-acid,*R*-AM isomer elutes after *R*-acid,*R*-AM.

The absolute configurations of (+)- and (−)-etodolic acid (ET) have not been reported. When reacted with *S*-(+)-AM, the (+)-ET,AM and (−)-ET,AM diastereoisomers have retention times of 17.08 and 18.75 min, respectively. Similar times are observed for the (−)-ET,*R*-(−)-AM (17.04 min) and (+)-ET,*R*-(−)-AM (18.69 min) isomers. If the *S*-configuration–dextrorotatory relationship (observed with the 2-APAs) also holds for ET, then the first-eluting isomers should have the *S*,*S* and *R*,*R* configurations when *S*-(+)-AM and *R*-(−)-AM, respectively, are the reactants. However, it must be recognized that ET is not a 2-APA and, in this respect, is not closely related to the other NSAIDs studied in this report. An additional difference is observed when comparing the extent to which the isomers are resolved, which, in the case of ET, is particularly noteworthy (approximately 2 min).

As with ET, the absolute configurations of the tiaprofenic acid (TA) enantiomers have not been reported. In addition, due to the higher priority of sulfur over oxygen (Cahn-Ingold-Prelog sequence rules for assigning *R*-, *S*-configuration [26]) the 2-APA enantiomers (compounds I—VII, Fig. 1) which have the *R*- and *S*-configurations would be assigned *S*- and *R*-, respectively, when describing TA isomers. Owing to the unavailability of TA enantiomers, we were unable to determine the configurational identity of the diastereoisomers. Reaction of racemic TA with *S*-(+)-AM gave GC peaks at 16.72 and 17.19 min and with *R*-(−)-AM at 16.80 and 17.25 min.

Structures of the diastereoisomeric amides were confirmed by electron-impact MS. With the exception of etodolic acid, which is not a 2-APA, the spectra of derivatized *S*- and *R*-ibuprofen (Fig. 5) are representative of the fragmentation patterns observed for the 2-APA diastereoisomers. Molecular ions were present in the mass spectra of derivatized ketoprofen, fenoprofen, naproxen, flurbiprofen and etodolic acid. The spectra contained a weak, but characteristic, $[M-117]^+$ fragment which arises by expulsion of a C_9H_9 radical

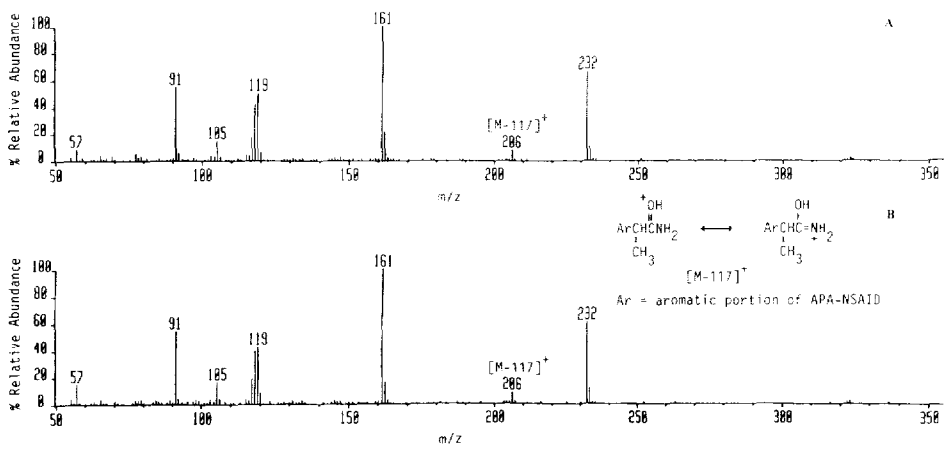

Fig. 5. Mass spectra of ibuprofen *S*-(+)-AM diastereoisomers. (A) *S*-ibuprofen,*S*-AM; (B) *R*-ibuprofen,*S*-AM.

from the amphetamine portion of the molecular ion. An appropriate structure for the $[M-117]^+$ fragment is shown in Fig. 5. Other characteristic fragments included an $[M-91]^+$ ion via the loss of a benzyl radical, and fragments of m/z 91 $[C_6H_5CH_2^+]$, m/z 119 $[C_6H_5CH_2CH^+CH_3]$, m/z 118 $[C_6H_5CH{=}CHCH_3^{+\cdot}]$, and m/z 117 $[C_6H_5C^+{=}CHCH_3]$ which are also derived from the amphetamine portion of the molecule.

The $[M-162]^+$ fragment is prominent and, with the exception of ketoprofen and flurbiprofen, is the base peak in all spectra. This ion is the result of benzylic cleavage of the NSAID portion of the molecular ion. In the case of ibuprofen, the resulting ion (m/z 161) is $[C_4H_9PhCH^+CH_3]$. The corresponding ions for the other NSAIDs are: ketoprofen (m/z 209), naproxen (m/z 185), fenoprofen (m/z 197), flurbiprofen (m/z 199), pirprofen (m/z 206, 208), cicloprofen (m/z 193) and tiaprofenic acid (m/z 215).

An abundant $[M-161]^{+\cdot}$ fragment is also observed and arises from the molecular ion by McLafferty rearrangement and expulsion of $C_6H_5CH_2CH(CH_3)N{=}C{=}O$. All derivatized 2-APAs gave this fragment; in the case of ibuprofen, the resulting ion (m/z 162) is $[C_4H_9PhCH_2CH_3]^{+\cdot}$.

Derivatized etodolic acid fragments in a different manner because of the influence of the oxygen-containing ring system. Diagnostic fragments present were the molecular ion, m/z 404, $[M-29]^+$, m/z 378 $[M-204]^+$, m/z 228 and m/z 214 derived from the etodolic acid portion of the amide, and the ion, m/z 91 ($C_6H_5CH_2^+$), arising from the amphetamine portion.

In conclusion, the method described is facile, sensitive and readily applied to the separation and quantification of anti-inflammatory arylalkanoic acid enantiomers in biological samples.

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