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Short communication

High-performance liquid chromatographic determination of primary aromatic amines in urine after derivatization to an azo dye with 2-aminoanthracene

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Abstract

A sensitive HPLC method for the determination of primary aromatic amines (anilino compounds) is described. Samples were prepared by derivatization of the substrate to an azo dye with 2-aminoanthracene (2-AA). 2-AA was found to react with the diazonium salts prepared from substituted anilines such as 4-halo, -sulfonyl, -carboxyl, -nitro or -acetyl derivatives, but not 4-hydroxy or -alkyl derivatives. In this work, three model compounds [sulfanilamide, 4-aminobenzoyl-β-alanine and 4-aminobenzoic acid (PABA)] were used to test the linearity and accuracy of the method. Chromatographic separation was carried out using a reversed-phase column (ODS) and ultraviolet detection at 279 nm. Good linearity for the three compounds was found within the range 50–2000 ng/ml. The intra-day coefficient of variation for the three compounds (at 100, 500, 1000 ng/ml) was below 10%. Using this method, the urinary excretion of PABA and its metabolites was studied after oral administration of PABA to rats.

1. Introduction

Many pharmaceuticals and chemicals contain a primary aromatic amine moiety, and an accurate method to determine these compounds is necessary. For such purposes HPLC has been widely used. However, this method has problems with water-soluble compounds of low concentration in biological fluids.

In spectrophotometric methods for primary aromatic amines, 1-(2-aminoethylamino)naphthalene [1] and 1-(2-diethylamino)naphthalene [2] have been used as derivatization reagents to form azo dyes. However, when the substrate contains an acid moiety [e.g., 4-aminobenzoic acid (PABA)], the corresponding azo dye is not well extracted by an organic solvent from aqueous phase because of its amphoteric nature. This problem makes the determination of such substrates by HPLC difficult.

This paper describes a simple HPLC method for the determination of primary aromatic amines using 2-AA instead of 1-aminonaphthalene derivatives [1,2] as an azo coupling reagent, because the azo dye derived from 2-AA

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is easily extracted by organic solvents. The sensitivity and specificity of this method make it suitable for monitoring the composition of biological samples such as plasma or urine.

2. Experimental

2.1. Materials

1-Aminoanthracene (1-AA) and 2-aminonaphthalene (2-AN) were obtained from Aldrich (Milwaukee, WI, USA), 2-AA and anilino compounds from Nacalai Tesque (Kyoto, Japan) and 1-(2-diethylaminoethylamino)naphthalene from Wako (Osaka, Japan). Methanol, acetonitrile and water were of HPLC grade.

2.2. Apparatus and chromatographic conditions

The HPLC apparatus (Hitachi–Waters) consisted of a Model 655A-12 pump, L-4000 UV spectrophotometric detector, D-2500 integrator and WISP 710A autosampler. The ODS reversed-phase chromatographic column (YMC-Pack A-312; 150 × 6 mm I.D., particle size 5 μm) was purchased from YMC (Kyoto, Japan). The mobile phase was methanol–water–acetic acid (78:22:1, v/v/v), and it was degassed ultrasonically before analysis. The flow-rate was maintained at 1.0 ml/min. The detection wavelength was set at 279 nm.

2.3. Validation procedure

The linearity and accuracy of the method were validated by adding various amounts of sulfanilamide (SA), 4-aminobenzoyl-β-alanine (4-ABA) and PABA as model compounds to human urine diluted with water (1:10). For testing the three compounds were dissolved in methanol, and 50 μl of this solution were added to 500 μl of diluted urine. The linearity was studied using urine spiked at 0, 50, 100, 250, 500, 1000, 1500 and 2000 ng/ml as final concentrations. The accuracy was tested at three different concentrations of the three compounds added to diluted urine (100, 500 and 1000 ng/ml, $n = 5$).

2.4. Sample preparation

To 500 μl of diluted urine, 50 μl of a methanolic solution of the three model compounds were added, followed by 100 μl of 0.5 *M* hydrochloric acid, and after addition of 100 μl of 0.1% sodium nitrite in water, the mixture was vortex mixed and left for 10 min. Subsequently, 100 μl of a 2% solution of ammonium sulfamate in water were added, and after a 15-min interval, 100 μl of 0.05% 2-AA in acetonitrile were finally added and the reaction mixture was left for 15 min. After addition of 2-AA, it is preferable to keep the solution in the dark.

Diethylether (5 ml) was added and the reaction mixture was shaken for 5 min. After shaking, the sample was centrifuged to separate the phases, and the upper phase (4 ml) was transferred into a glass tube for evaporation under vacuum. The residue was dissolved in 300 μl of methanol and an aliquot (30 μl) was injected into the chromatographic system.

2.5. Animal study

Seven-week-old male Crj-CD rats were used in biological experiments. The overnight-fasted rats received a PABA solution of 30 mg/kg, and urine was collected for 24 h. The determination of PABA and 4-aminohippuric acid (PAHA) was carried out according to the method described above.

Acetylated metabolites were extracted twice with ethyl acetate from acidified urine and the residue after evaporation of organic solvent was dissolved in 1 ml of 1 *M* hydrochloric acid, then hydrolysed at 90°C for 45 min. A solution of 5 *M* sodium hydroxide (0.18 ml) was added to the hydrolysate, then determination was performed according to the same procedure. The assay of the conjugate was carried out before and after treatment with glucuronidase.

3. Results and discussion

3.1. Reactivity of anilino compounds with 2-AA

Various anilino compounds were tested for their reactivity with 2-AA reagent. Good results were observed for compounds having an electron-withdrawing group, such as halo, sulfonyl, nitro, carboxyl or acetyl at the 4-position of the anilino nucleus. However, the anilines with an electron-donating group, such as hydroxy or alkyl, gave no diazo products.

As a reagent, 2-AA can be recommended especially for water-soluble compounds because the derivatization proceeds in water. The fluorescence of 2-AA was diminished after coupling. In the coupling reaction, the product yield reached the maximum 5–15 min after addition of 2-AA. If 2-AN was used instead of 2-AA in the same procedure, the coupling reaction proceeded similarly but slower.

Fig. 1 shows a typical chromatogram obtained after the coupling reaction of the three model compounds and 4-aminoacetophenone with 2-AA.

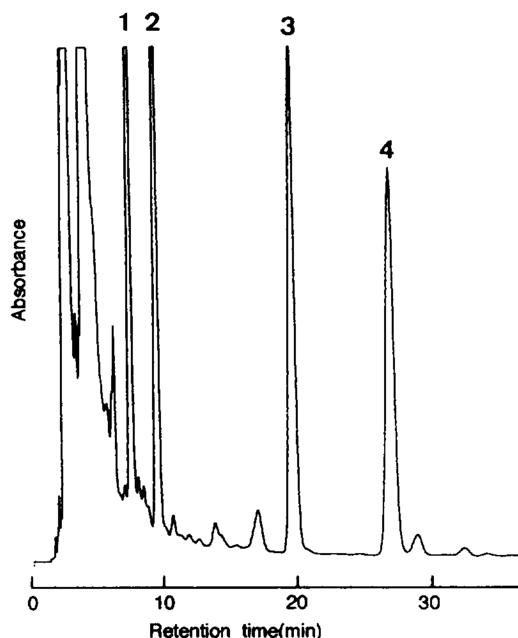

Fig. 1. Chromatogram of reaction products of model compounds with 2-aminoanthracene in diluted urine. Small peaks not assigned resulted from the degradation of the reagent (2-AA). Peaks: 1 = sulfanilamide (SA); 2 = 4-aminobenzoyl-β-alanine (4-ABA); 3 = 4-aminobenzoic acid (PABA); 4 = 4-aminoacetophenone. Total amount injected was 40 ng.

3.2. Extraction of azo dye

The azo dyes derived from 1-AA and substrates having an acid moiety, e.g., PABA or PAHA, were only slightly extracted with diethyl ether or ethyl acetate. Also, extraction was impossible with 1-(2-diethylaminoethylamino) naphthalene as described in the literature [2]. On the other hand, the azo-dyes derived from 2-AA were extracted easily by organic solvents.

The positions of the aromatic nucleus of 1-AA and 2-AA attacked by diazotized aniline compounds were considered to be the 4- and 1-positions, respectively. It is considered that the difference in the structures of these products influences their extractability from the aqueous phase into the organic phase.

3.3. Linearity

As shown in Table 1, the calibration graphs for the three compounds were linear in the range 50–2000 ng/ml with a good correlation coefficient. The minimum detectable concentration of the compounds was 10 ng/ml in diluted urine at a signal-to-noise ratio of 3.

3.4. Accuracy

The intra-day coefficients of variation (C.V.) calculated for concentrations of 100, 500 and

Table 1
Regression statistics of model compounds for the calibration graphs (range 50–2000 ng/ml; $n = 4$)

Substance	Calibration line[a]	Correlation coefficient
SA	$y = 0.427x + 29$	0.9986
4-ABA	$y = 0.402x + 21$	0.9987
PABA	$y = 0.296x - 1$	0.9994

[a] x = Absorbance; y = concentration (ng/ml).

Table 2
Accuracy of determination of model compounds in diluted urine using 2-aminoanthracene ($n = 5$)

Compound	Concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Intra-day C.V. (%)	Recovery (%)
SA	100	93 ± 6	6.5	93.0
	500	434 ± 26	6.0	86.8
	1000	997 ± 40	4.0	99.7
4-ABA	100	73 ± 1	1.4	73.0
	500	472 ± 28	5.9	94.4
	1000	927 ± 59	6.4	92.7
PABA	100	102 ± 5	4.9	102.0
	500	496 ± 18	3.6	99.2
	1000	987 ± 44	4.5	98.7

1000 ng/ml were 0.8–6.9%. The results are given in Table 2.

3.5. Metabolism of PABA in rats

The method was applied to rat urine. It has been reported [3] that after oral administration of PABA to rats, PABA and its metabolites were detected in rat urine. The results obtained are given in Table 3. The major metabolite was PAHA, and excretion of unchanged PABA was observed in smaller proportions. Further, PAHA and PABA were found to be excreted after acetylation, and the glucuronide of PABA was a minor metabolite.

Table 3
Urinary excretion of PABA and its metabolites after oral administration of PABA (30 mg/kg) to rats

Metabolites	Excretion (% of dose)[a]
4-Aminobenzoic acid	2.0 ± 0.4
4-Aminohippuric acid	44.5 ± 4.9
4-Aminobenzoyl glucuronide	2.3 ± 1.1
4-Acetamidobenzoic acid	27.7 ± 4.1
4-Acetamidohippuric acid	13.9 ± 1.2
Total	90.4 ± 6.0

[a] Mean ± S.D. ($n = 4$).

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