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SIMULTANEOUS DETERMINATION OF TRANILAST AND METABOLITES IN PLASMA AND URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method has been developed for the simultaneous determination of Tranilast, N-(3′,4′-dimethoxycinnamoyl)anthranilic acid (N-5′), and metabolites in plasma and urine from humans, dogs and rodents administered N-5′. Total N-5′ and metabolite N-3 conjugates were determined in human urine. Detection limits in plasma were 0.2 μg/ml for metabolite N-3-S and N-5′ and 0.1 μg/ml for metabolites N-3 and N-4. In urine, detection limits were 2 μg/ml for metabolite N-3-S and N-5′ and 1 μg/ml for metabolites N-3 and N-4. Metabolite N-4 was not identified in any sample assayed.

INTRODUCTION

Tranilast [N-5′ or N-(3′,4′-dimethoxycinnamoyl)anthranilic acid] is an anti-allergic, anti-asthmatic drug developed and marketed by Kissei (Japan) [1, 2]. The purpose of this paper is to describe a high-performance liquid chromatographic (HPLC) method for the simultaneous determination of N-5′ and metabolites (see Fig. 1) in plasma and urine. The method has been successfully applied for the determination of N-5′ and metabolites in human, dog and rodent plasma and urine.

EXPERIMENTAL

Materials

All reagents were analytical-reagent grade or better. Chromatographic solvents were HPLC grade. All mobile phase solutions were filtered prior to use

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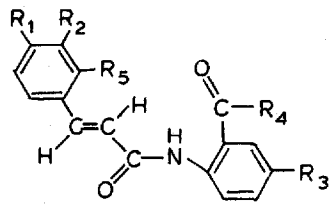

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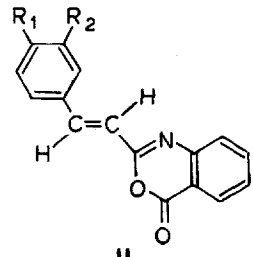

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Compound	Structure	R_1	R_2	R_3	R_4	R_5	Retention Time
N-5′	I	CH_3O	CH_3O	H	OH	H	11.7
N-5′-G	I	CH_3O	CH_3O	H	β-Glucuronide	H	
N-5′-U	I	CH_3O	CH_3O	H	Uronic Acid	H	
N-3	I	HO	CH_3O	H	OH	H	9.8
N-3-S	I	HO_3SO	CH_3O	H	OH	H	5.4
N-3-G	I	HO	CH_3O	H	β-Glucuronide	H	
N-4	I	CH_3O	OH	H	OH	H	10.2
IS	I	CH_3O	CH_3O	CH_3	OH	H	13.2
N-6	II	CH_3O	CH_3O				16.5
N-2	II	HO	CH_3O				15.9

Fig. 1. Structure of N-5′ and possible metabolites.

with Ultipor® NX 0.45-μm membrane filters (Rainin, Woburn, MA, U.S.A.). The ion-pairing reagent used in the isocratic determination of plasma was Waters Reagent A® (Waters Assoc., MA, U.S.A.) or tetrabutyl ammonium hydrogen sulfate (Regis, Morton Grove, IL, U.S.A.). SAX (strong anion-exchange) 500-mg extraction columns were supplied by Analytichem (Harbor City, CA, U.S.A.). Sep-Pak® octadecylsilane extraction cartridges (Waters Assoc.) were used to extract plasma and urine. Pellicular octadecylsilane (40 μm) was used to dry-pack direct-connect guard columns and was obtained from Scientific Systems (State College, PA, U.S.A.). Glusulase® was obtained from Endo Labs. (Garden City, NJ, U.S.A.). Control plasma was supplied by SeraTec Biologicals (North Brunswick, NJ, U.S.A.).

All standards were supplied by Merck Sharp and Dohme (Rahway, NJ, U.S.A.) and Kissei, with the exception of the internal standard (IS, see Fig. 1), which was synthesized in our laboratory [3]. Standards were dissolved in a small volume of N,N-dimethylformamide (DMF) and brought to the appropriate volume with 0.5 *M* sodium acetate, pH 6.

Apparatus

Plasma and urine were extracted using extraction cartridges and an Analytichem Vac-Elut® vacuum manifold. Sep-Pak cartridges were bridged to

the manifold using 200-μl pipet tips (any that can be used with Eppendorf or Pipetman pipettes) with the pointed end cut to fit the manifold. Plasma and urine hydrolysis samples were incubated in a Fisher Model 127 shaking water bath (Pittsburgh, PA, U.S.A.) set at 40°C.

The gradient HPLC separation was attained using a Series 4 liquid chromatograph, pumping at 1.5 ml/min, equipped with an ISS-100 autosampler (Perkin-Elmer, Woburn, MA, U.S.A.). The column system consisted of a direct-connect guard column (Applied Sciences, State College, PA, U.S.A.) and a Bioanalytical Sciences Biophase® octadecylsilane 5-μm, 25 cm × 4.6 mm analytical column (West Lafayette, IN, U.S.A.) held at 30°C by a Systec Goldenfoil column heating system (Minneapolis, MN, U.S.A.). Peaks were detected at 335 nm using a Kratos Spectroflow 757 UV detector (Westwood, NJ, U.S.A.). Data were generated by a Spectra-Physics SP-4270 integrator (Santa Clara, CA, U.S.A.). Gradient conditions were 0.05 *M* monobasic sodium phosphate, pH 5.3—methanol (70:30) linearly changing to 0.05 *M* monobasic sodium phosphate, pH 5.3—methanol (40:60) in 10 min with a final gradient to 100% methanol linearly changing in 3 min. Methanol was pumped for 4 min before returning to the initial conditions for 5 min prior to the next injection.

Isocratic data were collected using a Waters HPLC system consisting of a system controller, a WISP autosampler, and 6000A chromatography pumps. Peaks were detected using a Kratos Spectroflow 773 UV detector at 335 nm. Data were generated by a Spectra-Physics SP-4270 integrator. The column system included a Brownlee octadecylsilane 5-μm, 3 cm × 4.6 mm precolumn (Santa Clara, CA, U.S.A.) and an Analytical Sciences octadecylsilane 10-μm, 30 cm × 4.6 mm analytical column (Santa Clara, CA, U.S.A.). The mobile phase was pumped at 1.5 ml/min and consisted of methanol–0.05 *M* sodium acetate, pH 6 (55:45) with 10 ml of 0.5 *M* tetrabutyl ammonium hydrogen sulfate added to each liter.

Other columns evaluated included a Supelcosil® octadecylsilane 5-μm, 25 cm × 4.6 mm column (Supelco, Bellefonte, PA, U.S.A.), a Perkin-Elmer 3 × 3® octadecylsilane 3-μm, 3 cm × 4.6 mm short column and an Analytichem International Sepralyte® octadecylsilane 3-μm, 15 cm × 4.6 mm column.

Samples analyzed

The methods described have been applied to human, dog and rodent plasma and urine. Human subjects participating in a study to determine the pharmacokinetics of increasing single doses of N-5′ were administered 100, 500 and 1000 mg of N-5′ with a wash-out period between doses. Plasma and urine were collected at intervals throughout each dosing period and were determined for N-5′ and metabolites by the methods described. Beagle dogs were injected intravenously with N-5′ at a dose of 5 mg/kg. Urine and plasma samples were collected and determined for N-5′ and metabolites. Several rodent studies were conducted and biological fluids were determined for N-5′ and metabolites including plasma from mice given N-5′ by gavage, plasma from rats given N-5′ mixed with their food, and plasma and urine from rats injected intravenously with N-5′.

Sample preparation and extraction

Plasma. Plasma was prepared by placing 1.0 ml of human or dog plasma or 0.1 ml of rat or mouse plasma, 0.1 ml of internal standard (10 μg/ml) and 1.0 ml of 1 *M* sodium acetate buffer, pH 6, in a 12 × 75 mm polypropylene test tube. Prepared plasma was extracted on an octadecylsilane Sep-Pak cartridge that was prewet with 5 ml of methanol followed by 5 ml of 1 *M* sodium acetate buffer, pH 6. The plasma eluent was collected and passed through the Sep-Pak cartridge a second time. The cartridge was washed with 1 ml of 1 *M* sodium acetate buffer, pH 6, followed by 1 ml of 0.5 *M* sodium acetate buffer, pH 6. Excess buffer was removed from the cartridge with house vacuum and by washing with 0.2 ml methanol. The sample was collected from the cartridge with 1.0 ml methanol. Methanol was dried under nitrogen and the residue was reconstituted with 0.5 ml DMF and 0.5 ml water. After transferring to an autosampler vial, 25 μl were injected on the HPLC system.

Plasma hydrolyzed for the determination of metabolite N-4-S was prepared by placing 0.1 ml of plasma, 0.1 ml of internal standard (10 μg/ml), 0.1 ml of Glusulase (diluted 1:10 with 0.2 *M* sodium acetate, pH 4.9) and 0.7 ml of 0.2 *M* sodium acetate buffer, pH 4.9. The sample was incubated at 40°C for 1 h. After incubation, 1.0 ml of 1 *M* sodium acetate, pH 6, was added to the sample. Sample was extracted on Sep-Pak cartridges as described above.

Urine. Urine determined for free N-5′ and metabolites was prepared similarly to plasma. In a 12 × 75 mm polypropylene test tube, 0.1 ml of urine, 0.1 ml of internal standard (10 μg/ml) and 1.0 ml of 1 *M* sodium acetate buffer, pH 6, were added. After vortexing, the sample was extracted on an octadecylsilane Sep-Pak cartridge using the same method as for plasma.

Urine determined for total N-5′ and metabolite N-3 was hydrolyzed by Glusulase followed by base. The procedure evaluates free (N-5′ and metabolites observed in non-hydrolyzed samples), Glusulase-treated, and Glusulase-plus base-treated fractions. In a 12 × 75 mm polypropylene test tube, 0.3 ml of urine, 0.3 ml of internal standard (10 μg/ml) and 2.1 ml of 0.2 *M* sodium acetate, pH 4.9, were added. After vortexing, 0.9 ml was removed and placed in a test tube with 1.0 ml of 1 *M* sodium acetate, pH 6. This represents the free N-5′ and metabolites fraction (see Fig. 4B). The free fraction was prepared for chromatography using Sep-Pak cartridges, as previously described. To the remaining sample, 0.2 ml Glusulase (diluted 1:10 with 0.2 *M* sodium acetate, pH 4.9) was added. The sample was vortexed, capped and placed in a shaking water bath set at 40°C for 1 h. After incubation, 1.0 ml was removed and placed in a test tube containing 1.0 ml of 1 *M* sodium acetate, pH 6. This fraction represents free N-5′ and metabolites plus Glusulase-hydrolyzable conjugates (see Fig. 4C). This fraction was also extracted on Sep-Pak cartridges as previously described. The remaining sample was treated with 0.1 ml of 1 *M* sodium hydroxide and was incubated at 40°C. After 1 h, the sample was removed from the water bath, neutralized with 0.1 ml glacial acetic acid and extracted on a Sep-Pak cartridge. This fraction represents the total hydrolysis of urine and contains free plus Glusulase-hydrolyzable conjugates plus base-hydrolyzable conjugates (see Fig. 4D).

RESULTS AND DISCUSSION

A method for the simultaneous quantification of N-5′ and metabolites N-3-S, N-3 and N-4 in plasma and urine was presented. The method uses gradient HPLC with UV detection at 335 nm for the determination of N-5′ and metabolites in biological fluids. Metabolites N-2 and N-6, although extracted by the sample preparation procedure and detected under the HPLC conditions utilized, were not quantified owing to impurities present in authentic standards.

Several sample preparation methods were evaluated to isolate N-5′ and metabolites from plasma and urine. Protein precipitation of plasma by ethanol, 70% perchloric acid or acetonitrile, as well as liquid–liquid extraction by ethyl acetate, diethyl ether, methyl-*tert.*-butyl ether or methylene chloride, resulted in a poor recovery of N-5′ and/or metabolites.

In urine, strong anion-exchange (SAX) columns were used to isolate N-5′ and metabolites N-3-S, N-3 and N-4 from urinary substances as well as from N-2 and N-6. Separation of N-2 and N-6 was desired to eliminate their late elution and therefore decrease analysis time. Since N-2 and N-6 do not have ionizable functional groups, they should not be retained on the SAX columns. As is common with solid-phase extraction columns, more than one retentative property was involved; N-2 and N-6 were retained on SAX columns along with N-5′ and other metabolites. However, the strong retention to the SAX columns made it possible to remove many urinary interferents by washing columns with organic solvents and buffers over a wide pH range. In fact, retention was so strong that acidic methanol containing sodium chloride had to be used to recover analytes. Weaker anion-exchange columns did not retain analytes as effectively as SAX columns, resulting in a low recovery of N-5′ and metabolites. The method using SAX columns effectively isolated N-5′ and metabolites from urine but required a multi-step, time-consuming sample preparation with a 90% recovery of analytes.

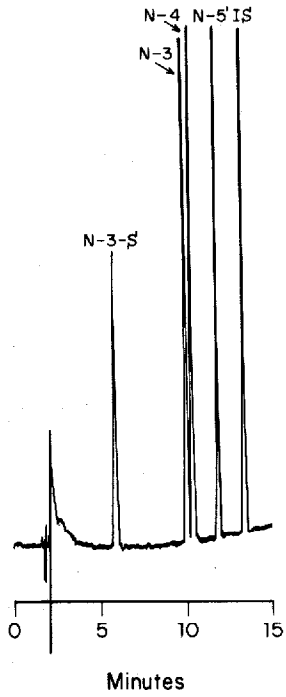
Sep-Pak octadecylsilane extraction of plasma and urine was the most efficient sample preparation method evaluated. By buffering Sep-Pak and samples with 1 *M* sodium acetate, pH 6, and by recycling samples, a 97% recovery of analytes was attained. The same method can be applied to both plasma and urine without co-extraction of interfering sample matrix components.

Plasma prepared by Sep-Pak extraction was originally chromatographed using an isocratic HPLC system (see *Apparatus*). Use of an ion-pairing agent added to the mobile phase, such as Waters Reagent A, effectively and selectively pulled N-3-S away from interfering peaks in the solvent front without affecting the retention time of other analytes. It was found that 10 ml of 0.5 *M* tetrabutyl ammonium hydrogen sulfate in 0.1 *M* sodium acetate adjusted to pH 7.5 added to 1 l of mobile phase was as effective as Waters Reagent A for ion pairing with N-3-S at less cost.

The isocratic system with the ion-pairing reagent in the mobile phase worked well for the determination of N-3-S, N-3 and N-5′ in plasma from human subjects administered 100-, 500- and 1000-mg doses of N-5′. When plasma from mice given a large dose of N-5′ was analyzed, several peaks were detected that

were not present in human plasma. Analysis of human urine was likewise difficult due to the presence of urinary interferences and additional drug-related peaks not observed in human plasma. Gradient-elution HPLC was required to resolve the observed peaks (Fig. 2).

Several columns were evaluated for the resolution of N-3-S, N-3, N-4, N-5', IS and other observed peaks. The Analytical Sciences column used in the isocratic HPLC system resulted in band broadening of peaks when samples were eluted using a gradient program with methanol as the organic modifier. Chromatography was improved using a column of similar length with 5-μm rather than 10-μm octadecylsilane packing. Fast liquid chromatography columns with even smaller packing material (3-μm octadecylsilane) resulted in no further improvement of chromatography. This can be partially attributed to the increase in column back-pressure observed and the inability to control the column back-pressure by heating the column. Heating of the short columns to 40°C resulted in poor peak shape and degradation of the column in a few days. Running at temperatures lower than 40°C and at a back-pressure compatible to the HPLC system did not decrease run times or improve chromatography when compared with the 5-μm octadecylsilane columns. Considering back-pressure, ability to resolve peaks of interest, and length of analysis time, the BAS 5-μm C_{18} octadecylsilane column was chosen for the gradient-elution HPLC determination of N-5' and metabolites. The analytical column was coupled



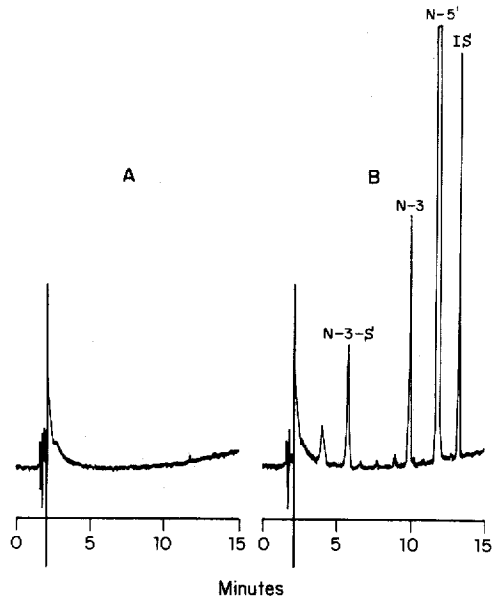


Fig. 2. N-3-S, N-3, N-4, N-5' and internal standard (IS) chromatographed using gradient-elution HPLC.

Fig. 3. Chromatogams of (A) pre- and (B) 3-h post-dosing plasma from a human subject administered 1000 mg N-5'.

TABLE I

VALIDATION DATA FOR THE DETERMINATION OF N-5', N-3-S, N-3 AND N-4 IN PLASMA AND URINE

Sample	Volume extracted (ml)	Analyte	Standard curve range (μg/ml)	Linearity* (r^2)	Reproducibility** (n = 6)	
					Intra-day	Inter-day
Plasma	0.1	N-5'	2.0—200	0.9999	2.7	4.9
		N-3-S	2.0—500	1.0000	3.5	5.6
		N-3	1.0—100	0.9999	2.5	8.0
		N-4	1.0—100	0.9999	2.3	5.1
Plasma	1	N-5'	0.2— 20	1.0000	1.5	3.6
		N-3-S	0.2— 20	1.0000	2.1	3.7
		N-3	0.1— 10	0.9999	2.4	2.9
Urine	0.1	N-5'	2.0—200	0.9999	0.8	3.4
		N-3-S	2.0— 50	1.0000	3.2	4.4
		N-3	1.0—100	0.9999	0.8	1.4
		N-4	1.0—100	0.9999	1.5	2.7

*Calculated by linear regression analysis of the mean values at each point of the standard curve for six daily runs.
**Expressed as relative standard deviation (%).

with a direct-connect guard column which offered several advantages over prepacked guard columns, including decreased back-pressure and the elimination of extra tubing between guard and analytical columns.

Validation data and detection limits for plasma and urine are presented in Table I. Detection limits in plasma were extended to 0.2 μg/ml for N-5' and N-3-S and 0.1 μg/ml for N-3 by extracting 1.0 ml of plasma. Calibration curves were constructed by spiking standards into blank plasma and urine at seven concentrations spread over the range of the standard curves listed in Table I. Quality control samples representing high and low points within the standard curve were prepared by spiking plasma or urine with each of the analytes of interest. Quality control samples were stored and treated identically as animal and human samples. Standard curves with quality control samples were prepared with each set of samples determined. Prior to determination of samples, standard curves plus quality control samples were assayed on six separate days to determine inter-day variation. Intra-day variation was determined by assaying six standard lines with quality control samples in one day. Results in Table I indicate the standard lines prepared were linear as r^2 values obtained from linear regression analysis exceeded 0.999. Intra- and inter-day reproducibility data presented demonstrates that the assay has little variation, with the relative standard deviation values less than 8%. The method was selective for N-3-S, N-3, N-4 and IS since no interfering peaks were observed in predose or control plasma or urine assayed (Figs. 3 and 4).

Quality control samples stored at −70°C were stable for at least six months. Plasma spiked with N-5' was stable for at least one month when stored at −20°C. After two months, an additional peak was observed, which has not

TABLE II

HUMAN PLASMA CONCENTRATIONS (μg/ml) OF N-3-S, N-3 AND N-5′ FOLLOWING ADMINISTRATION OF THREE DIFFERENT DOSES OF N-5′

Period	Dose (mg)	Metabolite	Time after administration of N-5′ (h)							
			0	1	2	4	8	12	24	48
1	100	N-3-S	N.D.*	N.D.	N.D.	0.25	N.D.	N.D.	N.D.	N.D.
		N-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
		N-5′	N.D.	3.9	12.1	10.6	5.3	3.0	0.8	N.D.
2	500	N-3-S	N.D.	N.D.	0.3	1.0	0.8	0.4	N.D.	N.D.
		N-3	N.D.	N.D.	0.6	0.7	0.2	N.D.	N.D.	N.D.
		N-5′	N.D.	37.0	70.4	51.2	32.6	22.3	10.9	4.4
3	1000	N-3-S	N.D.	N.D.	0.6	1.2	1.4	1.2	1.1	0.8
		N-3	N.D.	1.5	2.2	1.8	0.5	0.2	N.D.	N.D.
		N-5′	N.D.	128.8	112.7	92.8	70.5	56.4	35.1	21.1

*N.D. = Not detected under the detection limits set for the assay.

TABLE III

RECOVERY (mg) OF N-3-S, N-3 AND N-5′ IN HUMAN URINE FOLLOWING ADMINISTRATION OF THREE DIFFERENT DOSES OF N-5′

Urine was pooled over a 72-h collection period.

Period	Dose (mg)	Free			Glusulase-treated			Glusulase- plus base-treated		
		N-3-S	N-3	N-5′	N-3-S	N-3	N-5′	N-3-S	N-3	N-5′
1	100	N.D.*	4.9	15.0	N.D.	4.1	30.4	N.D.	4.5	34.9
2	500	17.2	19.5	42.5	N.D.	53.9	75.7	N.D.	59.1	87.0
3	1000	29.6	45.7	33.3	N.D.	137.7	138.7	N.D.	139.8	146.5

*N.D. = Not detected under the detection limits set for the assay.

been characterized. All samples were stored at - 70°C until time of analysis.

The described methods have been applied to the determination of free N-3-S, N-3, N-4, and N-5′ in plasma and urine from humans, dogs and rodents administered N-5′ and to the determination of total conjugates (free plus Glusulase-hydrolyzable plus base-hydrolyzable) in human urine. Table II presents the results of the determination of free N-5′ and metabolites in human plasma for a subject administered three different doses of N-5′ with a wash-out period between doses. Table III presents the results for free and total N-5′ and metabolites determined in urine collected from the same subject. Results are presented in milligrams recovered in urine collected over a 72-h collection period.

Total N-5′ and N-3 in urine was determined by hydrolysis with Glusulase and base. Both hydrolysis agents were necessary to determine total conjugates since N-3-S is hydrolyzed by Glusulase but not base and N-5′-uronic acid is hydrolyzed by base but not Glusulase. Other conjugates expected to be present

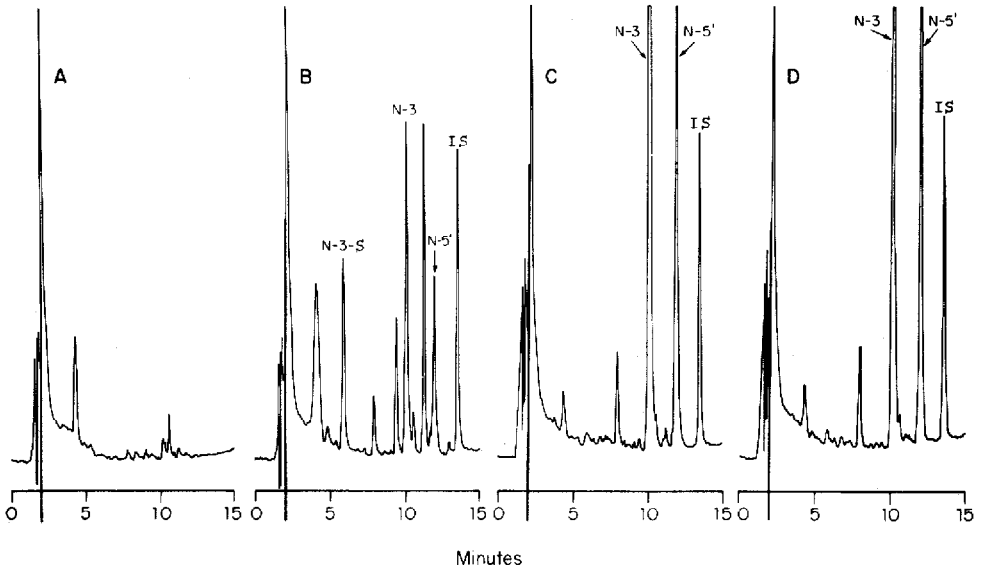


Fig. 4. Chromatograms of (A) pre- and (B—D) post-dosing urine pooled over 24 h from a human subject administered 1000 mg N-5′. Trace B represents extracted urine with no further treatment, trace C represents Glusulase-treated urine and trace D represents Glusulase-, then base-treated urine.

in urine are N-5′-β-glucuronide and N-3-β-glucuronide, both hydrolyzable by base or Glusulase.

The efficiency of hydrolysis for Glusulase and base was evaluated over a 24-h period using subject urine. No change in the amount hydrolyzed was noted after 1 h. Fig. 4 illustrates the chromatographic results of the stepwise hydrolysis of urine. Fig. 4A represents predose urine and emphasizes the absence of urinary substances in the areas where metabolite peaks elute. Figs. 4B—D represent successive treatment of the same urine sample showing free N-5′ and metabolites, free plus Glusulase-hydrolyzable conjugates and free plus Glusulase- plus base-hydrolyzable conjugates, respectively. The difference between Fig. 4B and 4C is due to the hydrolysis of N-3-S, N-3-β-glucuronide and N-5-β-glucuronide to N-3 or N-5′ by Glusulase. The lesser difference noted between C and D is probably due to the base hydrolysis of N-5′-uronic acid.

As has been demonstrated by the hydrolysis of urine, Glusulase is a useful tool. Another application of the developed method was to demonstrate that neither N-4 nor N-4-S were metabolites in human plasma. On the HPLC system used, N-3-S and N-4-S have the same retention time. To prove that the N-3-S peak was purely N-3-S and not a mixture of N-3-S and N-4-S, plasma was determined for free N-3-S (N-4-S), N-3, N-4 and N-5′. No N-4 was observed. Plasma was then treated with Glusulase to hydrolyze sulfate and β-glucuronide conjugates. Evaluation of the post-hydrolysis sample again showed no N-4. If N-4-S had been a metabolite, N-4 would have been observed following Glusulase treatment. Using the same rationale, neither N-4-S nor N-4 were metabolites in human urine.

A gradient HPLC method has been developed to simultaneously determine

N-3-S, N-3, N-4 and N-5′ in plasma and urine from humans, dogs or rodents administered N-5′. Application of the method to spiked plasma and urine, and clinical and animal plasma and urine, has shown the method to be reproducible and reliable.

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