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Note

Convenient method for the analysis of nicotinic acid as a metabolite of nicotinate esters in various tissue homogenates

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Nicotinic acid is a compound of considerable biological importance Besides belonging to the group of hydrosoluble B vitamins, it is also a drug possessing vasodilating, hypolipemic and fibrinolytic properties [1–7] Nicotinic esters are potential prodrugs of this agent, and a few such derivatives have indeed been described In our laboratory, a number of esters of nicotinic acid with various alkyl, arylalkyl and aryl substituents have been prepared [8] These nicotinates are to be used as substrates for carboxylesterases in various animal tissues with the goal of obtaining some insights into structure–metabolism relationships In such studies, very small amounts of the metabolite nicotinic acid must be extracted from complex biological media, e g brain homogenates, and quantified with good precision and accuracy without artifactual hydrolysis of the remaining substrate An efficient, sensitive, selective and reliable method of analysis is therefore required

Several methods have been reported for the determination of nicotinic acid in biological samples [9–14]. Analysis in urine samples by ion-exchange chromatography coupled with fluorescence detection was reported [9], but this method is not applicable since two of its steps would involve extensive chemical hydrolysis of some of the most labile nicotinates A sensitive high-performance liquid chromatographic (HPLC) method with UV detection was described [10] for the determination of nicotinic acid and its metabolites in aqueous media such as urine and plasma However, this method can hardly be

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applied to complex biological media such as tissue homogenates In another study [11], nicotinic acid was analysed in serum samples after derivatization to a fluorescent derivative, but the derivatization procedure required the medium to be heated in boiling water for 15 min, which must have resulted in extensive ester hydrolysis This method does not allow the processing of a large number of samples because fluorescent derivatives lack sufficient stability

An extensive literature survey revealed only very few HPLC methods allowing the determination of nicotinic acid in media more complex than plasma, serum or urine, but none satisfied all conditions Most methods [9,11,12] advocate a chloroform extraction to remove more lipophilic tissue components from the aqueous layer containing polar nicotinic acid Chloroform is not suitable to our purpose, as we wish to develop a single extraction procedure for nineteen nicotinic esters with log k_w^0 values ranging from 0 4 to 4 6 (i e a 10^4 range in lipophilicity), the more hydrophilic esters would not be extracted quantitatively with chloroform Direct injection of our samples into the HPLC column, as is recommended for plasma [13], cannot be considered when working with tissue homogenates Another method used to quantify nicotinic acid and nicotinamide in meat [14] lacked the required sensitivity

In this study, we present an analytical method suitable to the specific requirement of biotransformation studies Nicotinic acid (the metabolite) can be extracted in low concentrations from tissue homogenates The extraction procedure is simple and efficient and allows the separation of nicotinic acid from esters (as substrates) of different lipophilicity without their artifactual hydrolysis during sample conditioning

EXPERIMENTAL

Chemicals

Solvents and chemicals were of at least analytical or HPLC grade Nicotinic acid and *n*-decylamine were purchased from Fluka (Buchs, Switzerland), the buffers 3-morpholinopropane sulphonic acid (MPS) and tris(hydroxymethyl)aminomethane (Tris) from Merck (Darmstadt, F R G) and ethyl nicotinate and pyrazinoic acid from Aldrich Chemie (Steinheim, F R G) Phenyl nicotinate was synthesized in our laboratory by a published method [15] Methanol and acetonitrile were purchased from Romil Chemicals (Loughborough, U K.)

Preparation of biological material

Male Sprague–Dawley rats of body weight 180–200 g (Madorin Kleintierfarm, Fullinsdorf, Switzerland) were decapitated and their brains and livers removed Subcellular brain and liver fractions were prepared according to Walther et al [16] for brain and to De Duve et al [17] for liver These fractions were incubated with nicotinic ester at 37 °C in 10 m*M* Tris buffer (pH

7 4) At the end of incubation, 120 μl of 0 1 *M* hydrochloric acid were added to stop the enzymic reaction and denaturate proteins The internal standard pyrazinoic acid (120 μl of a solution in pH 7 4 Tris) was added to reach a final concentration of $1\,3\cdot10^{-4}$ and $8\,7\cdot10^{-5}$ *M* in liver and brain incubates, respectively The pH was adjusted to 7 4 with 75 μl of 10% sodium carbonate, the suspension centrifuged for 10 min at 1000 *g*, and the supernatant separated

Extraction procedure

The above supernatant was extracted on a disposable column prepacked with 3 ml of phenylsilane bound to silica gel (J T Baker SPE system, Phillipsburg, NJ, U S A) Preliminary tests showed that under the present conditions such columns could be used three times without decrease in efficiency The retained nicotinic and pyrazinoic acids were eluted with 5 ml Tris buffer (pH 6 9) The eluate was then reconcentrated almost to dryness in a rotary evaporator (Buchi, Flawil, Switzerland, water-bath temperature 35°C), and the residue was dissolved in methanol–water (1 1, v/v). The esters were retained on the column and could be eluted with acetonitrile if required.

Analysis of nicotinic acid

Nicotinic acid was analysed by reversed-phase HPLC The equipment was an Anacomp 220 system (Kontron, Zurich, Switzerland) equipped with an autosampler Type MSI 730 S LC, a guard-column filled with 25–40 μm Li-Chroprep (Merck), a 25 cm × 4 mm I D column filled with 5-μm Hypersil ODS (Knauer, Berlin, F R G) and a UV detector set at 262 nm. The mobile phase (flow-rate 1 0 ml/min) was a MPS buffer ($8\,6\cdot10^{-3}$, pH 7 4) containing 10% acetonitrile and $9\,3\cdot10^{-4}$ *M* *n*-decylamine Under these conditions, the retention times of pyrazinoic and nicotinic acid were 9 7 and 13 3 min, respectively

RESULTS

Method validation

Figs 1 and 2 show the chromatograms obtained with liver and brain microsomal extracts The smallest detectable concentration of nicotinic acid was found to be $2\cdot10^{-6}$ *M* (Fig 2), but quantitations were performed only for peaks corresponding to more than $4\cdot10^{-6}$ *M* to assure sufficient accuracy

Triplicates of six known concentrations of nicotinic acid were prepared and extracted as described Linearity was observed over the whole concentration range of nicotinic acid (see below), but this aspect of the method's validation was examined in more details in separate experiments The extraction procedure allowed the recovery of $87\,7 \pm 1\,3\%$ pyrazinoic acid and $86.3 \pm 0\,8\%$ nicotinic acid Repeated analysis of the same nicotinic acid solution gave the concentration of nicotinic acid with a relative standard deviation of 1 8% ($n=6$), which confirmed the reproducibility of the method

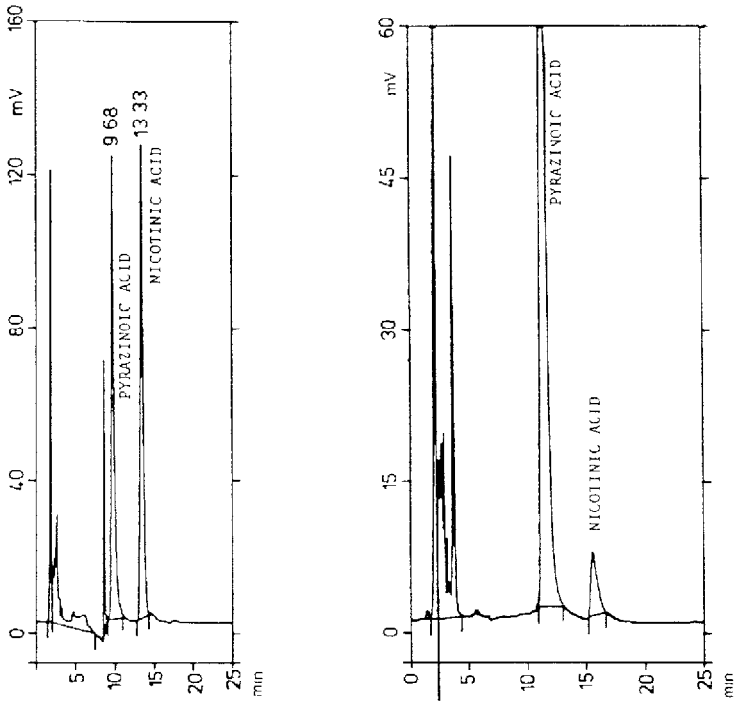

Fig 1 Chromatogram of nicotinic acid extracted from a liver microsomal incubation (pyrazinoic acid is the internal standard)

Fig 2 Chromatogram of nicotinic acid present in brain microsomes at a concentration of $4\ 10^{-6}$ *M* (pyrazinoic acid is the internal standard)

Simultaneous analysis of inactivated microsomes containing ethyl or phenyl nicotinate revealed no spontaneous formation of nicotinic acid during centrifugation and extraction At the end of the extraction, the nicotinate esters were no longer present in the samples, and no formation of nicotinic acid was to be feared prior to sample injection

Calibration curves

In accordance with literature values of esteratic activities in liver and brain, we established two calibration curves, one to quantify small amounts of nicotinic acid produced by brain fractions and the second to dose larger amounts of this acid in liver samples or in plasma

Calibration curve in liver fractions The assays were performed with nicotinic acid concentrations in the range from $1\ 75 \cdot 10^{-5}$ to $1\ 4 \cdot 10^{-3}$ *M*, yielding eqn 1 (95% confidence limits in parentheses)

$$y = 2\ 98(\pm 0\ 10)x + 2\ 88(\pm 7.01), \quad r^2 = 0\ 9996, \quad n = 7 \qquad (1)$$

TABLE I

HYDROLYTIC ACTIVITY OF RAT PREPARATIONS TOWARDS PHENYL NICOTINATE AND ETHYL NICOTINATE

Subcellular fraction	Activity[a]	
	Phenyl nicotinate	Ethyl nicotinate
Brain microsomes	1 92 ± 0 03	0 131 ± 0 011
Liver microsomes	689 ± 10	613 ± 6
Liver mitochondria	326 ± 4	154 ± 5
Brain mitochondria	1 37 ± 0 11	0 005 ± 0 001
Liver cytosol	78 4 ± 6 8	47 8 ± 3 0
Brain cytosol	0 76 ± 0 05	0 081 ± 0 007
Myelin	1 25 ± 0 09	0 094 ± 0 008
Plasma	22 0 ± 1 5	7 5 + 0 6

[a]Activities in nmol/min/mg protein (mean ± S D , $n=3$), measured at a substrate concentration of $1\,3 \cdot 10^{-4}$ M

where y is the nicotinic acid peak area expressed as a percentage of internal standard peak area, x is the nicotinic acid concentration in mol/l, r^2 is the squared correlation coefficient and n is the number of measurements

Calibration curve in brain fractions The assays were performed with nicotinic acid concentrations in the range from $4\,4 \cdot 10^{-6}$ to $2\,8 \cdot 10^{-4}$ M, yielding eqn 2

$$y = 4\,63(\pm 0\,17)x + 0\,88(\pm 2\,73), \quad r^2 = 0\,9993, \quad n = 6 \qquad (2)$$

Clearly excellent correlation coefficients were obtained in both cases The slopes of eqns 1 and 2 differ by a factor of 1 55 ± 0 12, which is not different concenfrom the ratio of internal standard concentrations in brain and liver fractions (1 49) The response was thus the same over the entire concentration range investigated (from $4\,4 \cdot 10^{-6}$ to $1\,4 \cdot 10^{-3}$ M) The relative errors (as expressed here by the 95% confidence limits) are 3 4 and 3 7% in the higher and lower concentration ranges, respectively

Biological application

Specific activities towards phenyl nicotinate and ethyl nicotinate are reported in Table I for rat plasma, liver and brain subcellular fractions It is worth noticing that this method has now been used successfully to quantify the enzymic hydrolysis of nineteen different nicotinate esters

DISCUSSION

Several methods to denaturate proteins and stop an enzymic reaction have been reported Heat treatment cannot be used owing to the thermolability of

esters Organic solvents are frequently used, but alcohols and particularly methanol had to be discarded because of known reactions of transesterification [18] Double peaks of nicotinic acid occurred in acetonitrile, and the large volume of acetone required for protein precipitation results in sample dilution Heavy metal salts (e g lead acetate or nitrate) and ammonium sulphate could not stop the reaction completely and produced parasite peaks in the chromatograms Basic denaturation with sodium hydroxide was tested, resulting in unacceptable saponification of esters We finally chose acidic denaturation of proteins with 0 1 *M* hydrochloric acid The pellet formed after addition of the acid and centrifugation was resuspended and found to be totally devoid of esteratic activity

Choosing an internal standard proved rather difficult A large number of molecules chemically related to nicotinic acid were tested, and pyrazinoic acid was finally selected for its hydrophilicity and good extractibility

To obtain greater precision, we were interested in quantifying the appearance of nicotinic acid, the reaction product, rather than the disappearance of the substrate This restricted the number of suitable extraction solvents, as nicotinic acid is soluble only in water and lower alcohols However, neither could be used as they may cause ester hydrolysis or transesterification A solid-phase extraction system was therefore preferred Among the several phases investigated, silica gel-bound phenylsilane was selected since it retains polar compounds such as nicotinic and pyrazinoic acid as well as lipophilic compounds such as our esters, but allows their separate elution

The mechanism governing the separation of our samples on the HPLC column is based on the formation of ion pairs [19] between nicotinic acid or pyrazinoic acid and *n*-decylamine Formation of the ion pairs dramatically increases the retention times compared with those of the parent acids, allowing a final separation of the two acids from other constituents and biological contaminants

Quantitative validation showed that the present method has sufficiently good sensitivity (limit of detection $2 \cdot 10^{-6}$ *M* nicotinic acid in incubates) for our metabolic studies of esteratic activity (where concentrations slightly higher than $2 \cdot 10^{-6}$ *M* are to be analysed) It is also conveniently simple and is now successfully applied to the determination of the kinetic constants of enzymic hydrolysis of a wide range of nicotinate esters in various biological preparations

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