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QUANTITATION OF POLYAMINES IN CULTURED CELLS AND TISSUE HOMOGENATES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THEIR BENZOYL DERIVATIVES

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SUMMARY

A rapid and simple method, originally described by Redmond and Tseng [J. Chromatogr., 170 (1979) 479] was applied to the analysis of di- and polyamines in cultured human tumour cells and human tumour xenografts. Optimization of the procedures and evaluation of the characteristic features of the assay are described. The (modified) procedure employs precolumn derivatization with benzoyl chloride, extraction of the derivatives by chloroform, separation by reversed-phase high-performance liquid chromatography under isocratic conditions and detection by ultraviolet absorbance measurement at 229 nm. The complete analysis was accomplished within 10 min per sample The detection limit was ca. 1 pmol. The intra- and inter-assay coefficients of variation were 2.5–4.4% and 3.4–13.1%, respectively. The presence of well known inhibitors of polyamine biosynthesis, such as DL-α-difluoromethylornithine and methylglyoxal bis(guanylhydrazone), did not interfere with the assay, and disturbance by cyclohexylamine could be avoided by changing the polarity of the mobile phase. The method proved to be very suitable because it is rapid, simple, requires a minimum of sample pretreatment, and still provides sufficient sensitivity to quantitate polyamines in relatively small amounts of cells (10^5 cells) or tumour tissues (less than 1 mg), even after treatment with inhibitors of polyamine biosynthesis.

INTRODUCTION

Polyamines are aliphatic polycationic compounds that are present in virtually all living cells. The natural polyamines–spermidine, spermine and their diamine

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precursor putrescine (1,4-diaminobutane)–usually occur within the cells mainly as unconjugated molecules. Monoacetyl derivatives, in particular of putrescine and spermidine, are the major urinary excretion forms but generally do not accumulate in tissues with the exception of certain pathophysiological conditions [1,2].

Although the precise physiological role of polyamines is not quite clear yet, their involvement in the regulation of cell proliferation is well established [3–6]. Lowering of intracellular polyamine concentrations, induced by treatment with polyamine synthesis inhibitors, was shown to result in decreased rates of proliferation in a variety of experimental tumour systems [7,8]. Investigations on the mechanisms of regulation and the role of each of the different polyamines in this process require a reliable method for the quantitation of intracellular polyamine concentrations under various conditions.

As reviewed recently by Seiler [9], a large number of chromatographic techniques have been applied for the determination of polyamine levels. At present the most widely used procedures employ high-performance liquid chromatography (HPLC) of fluorescently labelled derivatives, commonly obtained by reaction with dansyl chloride [10–12], or separation by HPLC followed by postcolumn derivatization with fluorescent ligands such as *o*-phthalaldehyde [13,14]. Alternatively, precolumn derivatization with UV-absorbing reagents has been applied. Redmond and Tseng [15] have introduced the use of benzoyl chloride for that purpose. This procedure was subsequently modified and applied to determine also the concentration of dicyclohexylamine [16], an inhibitor of spermidine synthase [17,18], in addition to the levels of polyamines. In this paper we report on the measurement of polyamine concentrations in cultured human prostate tumour cells and in tumour xenografts in nude mice by the application of the benzoylation procedure. In particular, several characteristics of the assay method, such as linearity, reproducibility and sensitivity, are described. Moreover, the efficiency of derivatization and extraction and possible interferences by a number of other compounds were evaluated. The effect of inhibitors on the polyamine content of tumour cells was investigated by means of the optimized procedure.

EXPERIMENTAL

Chemicals

All diamines and polyamines, including the acetyl-conjugated compounds, used for the preparation of standard solutions were purchased from Sigma (St. Louis, MO, U.S.A.). Methylglyoxal bis(guanylhydrazone) (MGBG) and cyclohexylammonium sulphate (CHAS) were also purchased from Sigma. Benzoyl chloride was obtained from Aldrich (Brussels, Belgium). DL-α-Difluoromethylornithine (DFMO) and (2*R*,5*R*)-6-heptyne-2,5-diamine (MAP) were generous gifts from the Merrell-Dow Research Institute (Strasbourg, France and Cincinnatti, OH, U.S.A.). Chloroform (spectroscopic grade, Uvasol), methanol (HPLC-grade, LiChrosolv), sodium hydroxide (Art. No. 9136) and all other inorganic reagents were obtained from E. Merck (Darmstadt, F.R.G.).

Instruments

Chromatographic analyses were carried out with a Kratos analytical liquid chromatographic system (Kratos Analytical, Ramsey, NJ, U.S.A.), equipped with a Spectroflow 450 solvent programmer, two Kratos Spectroflow 400 solvent-delivery system pumps, a Kratos Spectroflow 480 injector and a Kratos Spectroflow 783 programmable UV absorbance detector. Calculations were performed by a D2000 Merck chromato-integrator.

Sample preparation

The continuous cell line PC-93, established from human prostate cancer tissue, was used either after in vitro culture [19] or after growth in athymic nude mice [20]. In some experiments cell cultures were treated with DFMO or MAP, both inhibitors of ornithine decarboxylase [21,22], or with CHAS, an inhibitor of spermidine synthase [17,18], in order to study the effect of polyamine synthesis inhibitors on the polyamine concentrations in these cells. DFMO was used also in the in vivo experiments. For that purpose the host animals were injected intraperitoneally with DFMO daily at a dose of 500 mg/kg body weight; in addition the drinking fluid contained 2% (w/v) DFMO [23].

For the analysis of polyamine levels monolayers of cultured cells were washed with phosphate-buffered saline. Cells were harvested by scraping and counted in a haemocytometer. To the cell pellet obtained after centrifugation an appropriate amount of internal standard, 1,6-hexanediamine (10 nmol per 1–2 million cells) and 1 ml of 0.3 *M* perchloric acid (PCA) were added. Weighed aliquots of (tumour) tissues were homogenized in 5–10 volumes of cold 0.3 *M* PCA in the presence of a known amount of internal standard, using a Potter-Elvejhem homogenizer. The clear supernatants obtained after centrifugation of PCA-treated samples were stored frozen ($-20°C$) until further analysis was carried out.

Derivatization procedure and extraction of benzoyl polyamines

In glass tubes (Kimax culture tube, 16×100 mm) 2 ml of 2 *M* sodium hydroxide and 10 μl of benzoyl chloride were added to 100–400 μl of the PCA extract. Tubes were closed with caps provided with PTFE-coated rubber closures, and the contents were mixed vigorously and incubated for 30 min at room temperature under gentle rotation. Subsequently the incubation mixture was extracted with 2 ml of chloroform. After centrifugation the chloroform phase was removed, washed with 2 ml of HPLC-grade water, centrifuged and transferred to another tube. The solvent was evaporated under a stream of air and the residue was resuspended in 100–500 μl of methanol.

Chromatographic conditions

Separations were performed at ambient temperature using two ChromSep HPLC columns (100 mm×3.0 mm I.D.) packed with ChromSpher C_{18} reversed-phase material (5-μm particles), protected by a reversed-phase guard column (Chrompack International, Middelburg, The Netherlands). Volumes of 2–10 μl of the solution of derivatized compounds in methanol were injected onto the column. The mobile phase was methanol–water (usually 65:35, v/v, see Results).

Carbon-pretreated deionized water, filtered over a Norganic TM cartridge (Millipore, Bedford, MA, U.S.A.) to remove organic contaminants and de-aerated with helium gas, was used for preparation of the mobile phase. The flow-rate was 0.4 ml/min.

RESULTS

Optimal conditions of derivatization and extraction of derivatives

The efficiency of the derivatization and the extraction was investigated by the use of radioactively labelled polyamines. As the underivatized compounds were not extracted into the organic solvent layer to an appreciable extent, the amount of extractable radioactivity reflected the formation and extraction of benzoyl-conjugated polyamines. When 10 nmol of each of the polyamines were present in the incubation mixture, complete extraction of the derivatives was accomplished if at least 2 μl of benzoyl chloride (corresponding to a molar ratio of 1:500) were used for derivatization (Fig. 1). At low benzoyl chloride concentrations all non-extractable radioactivity was retraced in the aqueous phase; adsorbance of polyamines to the glass surface of the tubes was not detectable. Vigorous mixing of the reagent solution (carried out by vortexing and/or manual shaking), which resulted in the emulsification of benzoyl chloride, was found to be of critical importance to achieve optimal derivatization.

From these results it was decided to use 10 μl of benzoyl chloride for routine analyses. Under that condition the efficiency of derivatization and extraction did not decrease when the amount of polyamines in the sample was increased to 1000 nmol. The addition of saturated sodium chloride solution before the extraction [15] did not affect extractability and was, therefore, omitted. With regard to the solvent used for extraction it was observed that the use of chloroform, instead of

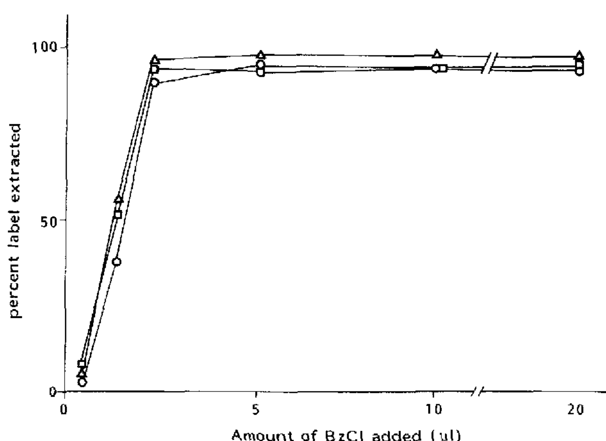

Fig. 1. Extractability of polyamines after derivatization with various amounts of benzoyl chloride. Radiolabelled polyamines (10^5 dpm) were added to standard mixtures containing 10 nmol of each of the polyamines All values, indicating the percentage of radioactive label extracted into the chloroform phase, represent the means from three determinations, S D. values were within the size of the symbols: ○ = putrescine; △ = spermidine; □ = spermine.

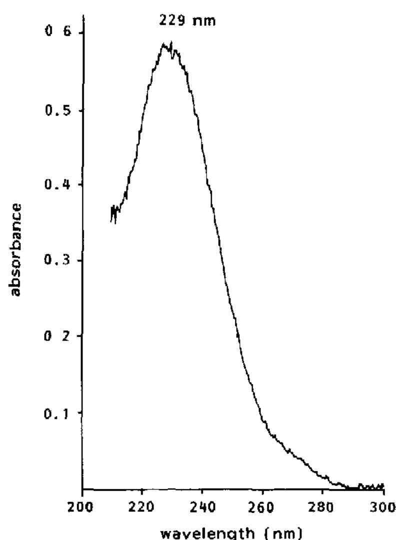

Fig. 2. Absorbance spectrum of purified benzoyl putrescine dissolved in methanol and measured against methanol as the blank.

diethyl ether [15], enhanced the efficiency of the extraction by ca. 20%. From other experiments, not employing radiolabelled compounds, evidence was obtained that benzoyl derivatives of acetylated polyamines were extracted by chloroform but not by diethyl ether (see below).

Characterization of benzoyl derivatives

By performing the derivatization procedure on a preparative scale, benzoylated polyamines were prepared in order to investigate their physicochemical properties. The products obtained after derivatization and extraction were extensively purified by repeated recrystallization from methanol–water. Purity was confirmed by HPLC analysis. The UV absorbance spectrum of benzoyl derivatives was completely identical with that of unconjugated benzoyl chloride, with a major peak at 229 nm (Fig. 2), and they were very stable at various temperatures (up to at least 100°C) even under acidic conditions (6 *M* hydrochloric acid).

In contrast to unbenzoylated polyamines (including monoacetyl polyamines) the benzoyl derivatives did not react with *o*-phthalaldehyde, which demonstrated the absence of primary amine groups in the benzoylated compounds. The number of benzoyl groups conjugated to each polyamine molecule was determined after acid hydrolysis and the subsequent measurement of both liberated polyamines and benzoic acid. HPLC analysis showed that hydrolysis was complete after heating for 16 h at 120°C in 12 *M* hydrochloric acid. Polyamines were quantitated after benzoylation (with 1,6-hexanediamine as the internal standard). Benzoic acid was quantitated directly in the diluted hydrolysate in the presence of a fixed amount of purified benzoylated 1,8-octanediamine. Under the conditions described for the chromatographic separation of polyamines, benzoic acid appeared as a sharp peak with a retention time of 4.5 min (mobile phase, methanol–water,

60:40, v/v); peak-area ratios (relative to the internal standard) were proportional to the benzoic acid concentrations over the whole range tested (0–1 mM). The results showed that the ratios of benzoic acid to polyamine were 2.1 ± 0.2, 2.8 ± 0.3 and 3.8 ± 0.3 (mean $\pm$ S.D. from three separate determinations) for benzoyl putrescine, spermidine and spermine, respectively, which indicates that all available amine groups were subject to benzoylation. Using the theoretical molecular mass values of fully benzoylated polyamines, the molar absorption coefficients were calculated from the data obtained by UV spectroscopy of preweighed amounts of the purified compounds. Although the molar absorptivity increased with the number of amine groups (putrescine 17 300 M^{-1} cm^{-1}; spermidine 21 700 M^{-1} cm^{-1}; and spermine 28 900 M^{-1} cm^{-1}) some degree of hypochromicity (relative decrease of absorption intensity, presumably due to interaction of neighbouring transition dipoles) was observed with the larger numbers of benzoyl groups, so that a strict linear relationship did not exist.

Chromatographic separation of derivatized polyamines

Benzoylated polyamines were separated under isocratic conditions using a methanol–water mixture as the mobile phase. The time required to achieve complete analysis depended on the composition of the solvent mixture. Typically, analysis was completed within 10 min using 65% methanol and within 15 min using 60% methanol. The chromatogram for the separation of a standard mixture of putrescine, 1,6-hexanediamine (internal standard), spermidine and spermine is shown in Fig. 3. The area under the peak was of the same order of magnitude for each of the polyamines when present in equimolar concentrations. The observed differences, expressed as the ratio relative to the internal standard peak, were found to be consistent (putrescine 1.00; spermidine 1.09; spermine 1.22). Standard mixtures were always used for calibration prior to the analysis of unknown samples.

Retention times of derivatized polyamines and possibly interfering substances

The retention times of benzoylated polyamines were dependent on the composition of the mobile phase (Table I). Satisfactory separation of the main polyamines, including 1,6-hexanediamine as the internal standard, was achieved using methanol–water (65:35) (Fig. 3 and Table I). The peaks were separated even further by a mobile phase containing 40% water in methanol (Table I). Under both conditions the acetylated polyamines were separated as well, although the peaks of N^1- and N^8-acetylspermidine were not resolved from each other. The peak areas were consistently smaller than those of the corresponding unacetylated compounds. Interestingly, it was observed that benzoylated acetyl polyamines could not be extracted by diethyl ether.

The presence of unchanged benzoyl chloride might lead to a peak that coincides with either the spermidine or the internal standard peak (Table I). Under the usual incubation conditions, however, the excess benzoyl chloride was fully converted into benzoic acid, which did not interfere with any of the peaks of interest (retention times shown refer to standard conditions; in acidic media, as mentioned above, benzoic acid is eluted later). S-Adenosylmethionine turned out to

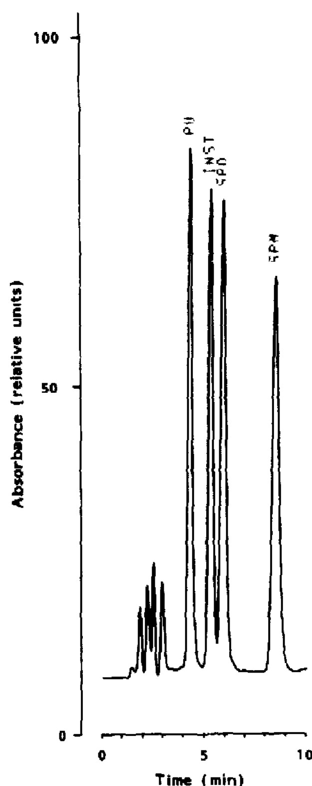

Fig. 3. Chromatogram for the separation of benzoylated putrescine (PU), 1,6-hexanediamine (internal standard, INST), spermidine (SPD) and spermine (SPM), monitored by their absorbance at 229 nm. A standard mixture containing 1 nmol per 0.1 ml was used for derivatization. Elution with methanol–water (65:35, v/v). Each of the peaks shown represents ca. 100 pmol of polyamine injected.

be a poor substrate for benzoylation. In spite of that, a (minor) peak was eluted close to spermidine, in addition to another peak at 2.2 min representing the underivatized compound. The benzoylated substance will not readily interfere with the quantitation of spermidine, however, as it represented only a few percent of the total amount used in the incubation; moreover, the benzoylated compound turned out to be rather unstable.

As metabolic inhibitors are widely applied in polyamine research, it was investigated whether the presence of such inhibitors did interfere with the analysis of polyamines. No peaks were observed with DFMO and MGBG (Table I). Two other inhibitors, MAP (a derivative of putrescine and inhibitor of ornithine decarboxylase) and CHAS (an inhibitor of spermidine synthase) were detectable after benzoylation. The peak of CHAS seriously interfered with spermidine when a mobile phase consisting of 35% water in methanol was used. However, as reported earlier by Porta et al. [16], benzoyl-CHAS was well separated from the benzoylated polyamines when water–methanol (40:60) was used. Under these conditions CHAS concentrations could be quantitated [16]. The peak-area ratio of CHAS relative to the internal standard was 1.0 when both compounds were

TABLE I

RETENTION TIMES OF BENZOYLATED POLYAMINES AND RELATED COMPOUNDS

N.D. = not detected.

Compound	Retention time (min)	
	Water–methanol (35:65)	Water–methanol (40:60)
Benzoic acid	2.38	2.50
N^1-Acetylputrescine	2.96	3 21
N^1-Acetylspermidine	3.63	4.33
N^8-Acetylspermidine	3.64	4.39
Putrescine	4.18	5.27
1,3-Propanediamine	4.43	5.64
N^1-Acetylspermine	4.48	6.52
Cadaverine	4.54	5.86
(2*R*,5*R*)-6-Heptyne-2,5-diamine (MAP)	4.78	6.38
1,6-Hexanediamine	5 18	7.24
Benzoyl chloride	5.79	7.42
Spermidine	5 80	8.82
S-Adenosylmethionine	5.83	8.29
Cyclohexylamine (CHAS)	5.94	7.96
1,7-Heptanediamine	6.22	9.38
1,8-Octanediamine	8.10	13.51
Spermine	8.36	15.60
α-Difluoromethylornithine (DFMO)	N.D.	N.D.
Methylglyoxal bis(guanylhydrazone)	N.D.	N.D.

present at equimolar concentrations (1 mol of CHAS contains two CHA^+ ions). MAP might be quantified by this procedure as well. The MAP peak was resolved from the other polyamine peaks, even when 35% water in methanol was used, and the peak area was proportional to the MAP concentration. The peak-area ratio of MAP relative to putrescine was consistently found to be ca. 0.5, indicating that only one benzoyl group was conjugated to MAP. The contribution of the allenyl group to the absorption at 229 nm appeared to be of minor importance (molar absorptivity 1750 M^{-1} cm^{-1}); measurement at 254 nm resulted in an only slightly lower peak-area ratio.

Blanks, consisting of tissue or cell homogenates incubated without benzoyl chloride or of reagents incubated in the absence of tissue extracts, did not reveal any peaks that could interfere with the quantitation of the usual polyamines.

Linearity of the assay and detection limits

The linearity of the assay was investigated by incubating various amounts of polyamines in the presence of a constant amount of internal standard (10 nmol of standard added to incubation mixtures containing 1–100 nmol of polyamine; 1 nmol of standard added to 0.05–1.0 nmol polyamine). A linear relationship between the polyamine concentration and the peak-area ratio relative to the in-

ternal standard existed over the entire range (up to 100 nmol); the average linear regression coefficient was greater than 0.97.

In the experiment described above an amount of 0.05 nmol in the incubation mixture could still reliably be quantitated. Assuming that all polyamines were quantitatively recovered in the methanol solution, this amount corresponded to 2.5 pmol of polyamine injected onto the column. Signal-to-noise ratios were determined after further lowering the amount of polyamines injected. It was found that the injection of 1 pmol resulted in a signal-to-noise ratio of ca. 4. Therefore, the minimum detection level should be considered to be ca. 1 pmol.

Measurement of polyamine concentrations in cells and tissues

Polyamines were extracted from cells and tissues by treatment with 0.3 *M* PCA. Omission of the deproteinization step (i.e. by using homogenates in phosphate buffer instead of PCA) usually resulted in the appearance of additional, unidentified peaks in the chromatogram. Experiments in which radiolabelled polyamines had been added to cell cultures (1–4 h prior to harvesting) indicated that the polyamines were quantitatively extracted (more than 99%) by PCA treatment.

The chromatogram for the separation of benzoylated polyamines from PC-93 prostate tumour tissue grown in nude mice is shown in Fig. 4. In comparison with untreated controls (Fig. 4A), tumour tissues obtained from animals treated with DFMO for seven days (Fig. 4B) contained considerably lower concentrations of spermidine and, in particular, of putrescine (which was barely detectable in treated tumour tissues), whereas spermine levels remained almost unaffected. Quantitative data are shown in Table II. The chromatograms were not improved further by prepurification of the samples by published procedures employing selective adsorption onto and elution from Sep-Pak cartridges [24]. In fact, the (unnecessary) prepurification step sometimes resulted in selective losses of diamines in comparison with spermidine and spermine. Omission of this step therefore also eliminated the need for the addition of multiple internal standards [24].

Similar to the results obtained with xenografts, exposure of monolayer cultures of PC-93 to DFMO mainly caused a reduction of putrescine and spermidine levels (Fig. 5A and B), but, as expected, the inhibition was achieved more readily (within four days) and more completely (in particular for spermidine; see Table II) with tumour cells in vitro. When expressed in nmol per mg DNA, polyamine levels were of the same magnitude in cultured cells and in xenografts.

Fig. 5C shows the effect of inhibition of spermidine synthase by CHAS in cultured PC-93 cells. In addition to a substantially lower peak for spermidine and an increased putrescine peak, CHAS could also be detected (and quantitated), provided that the polarity of the mobile phase was properly adjusted (cf. Table I).

Recovery, precision and reproducibility

To determine the analytical recovery, 20 nmol of polyamines were added to fixed amounts of tissue homogenate (containing 1.5, 10 and 13 nmol of putrescine, spermidine and spermine, respectively) and the increase of the concentrations was measured for each individual polyamine. The recoveries, as determined

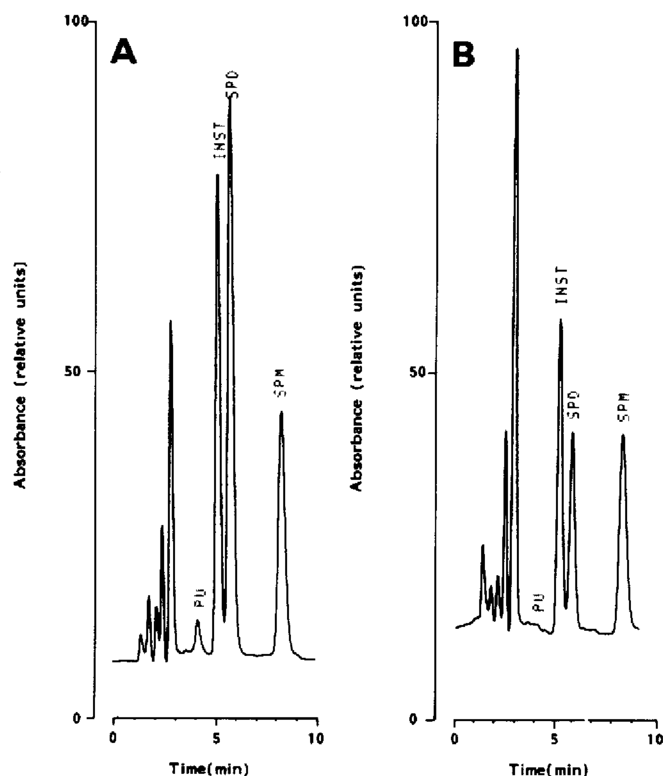

Fig. 4. Separation of benzoylated polyamines from PC-93 tumour tissue grown in nude mice. Tissue extracts were treated with 0.3 *M* perchloric acid. Peak labels as in Fig. 3. Elution with methanol-water (65:35, v/v). (A) Untreated control; (B) tumour tissue from a nude mouse treated with DFMO for seven days by oral administration (2% DFMO in drinking water) plus intraperitoneal injection (500 mg/kg).

TABLE II

POLYAMINE LEVELS IN XENOGRAFTS AND CULTURED PC-93 TUMOUR CELLS

	Polyamine level		
	Putrescine	Spermidine	Spermine
Xenografts (nmol/mg tissue)			
Untreated	0 054 ± 0 017	1.05 ± 0.10	0.60 ± 0.07
DFMO*	0.003 ± 0.004	0.53 ± 0.02	0.61 ± 0.05
Cultured cells (nmol/10⁶ cells)			
Untreated	0.39 ± 0.08	2.03 ± 0.13	1.90 ± 0.03
DFMO*	0.06 ± 0.04	0.32 ± 0.12	1.64 ± 0.56

*Nude mice were treated with DFMO for seven days by oral administration (2% DFMO in drinking water) plus intraperitoneal injection (500 mg/kg) Cells were cultured in the presence of 1 m*M* DFMO for four days.

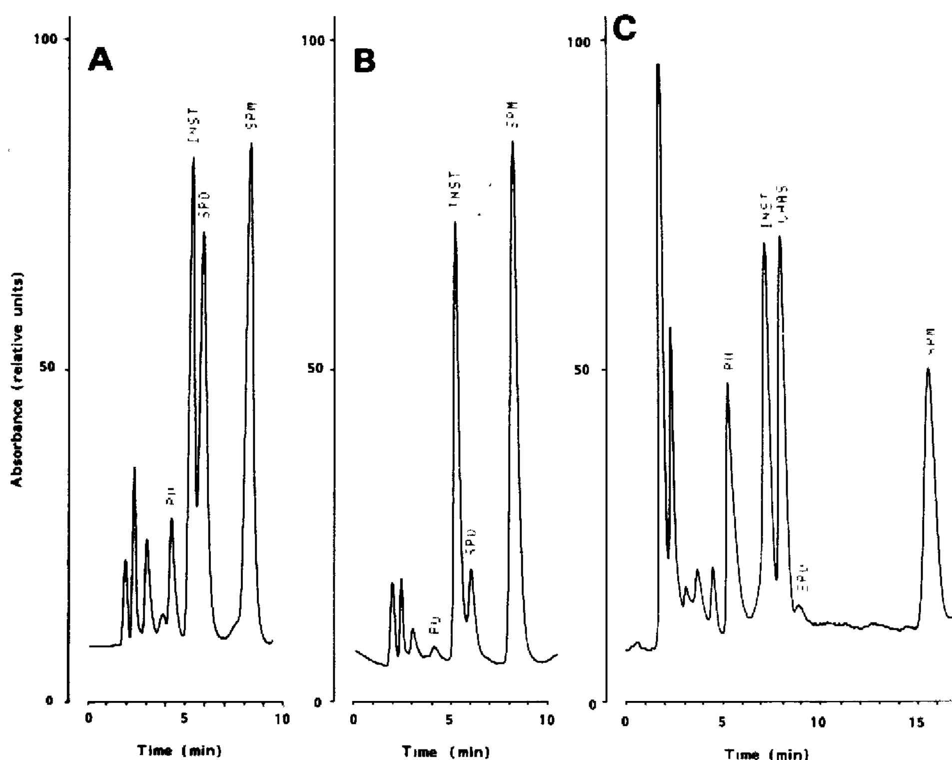

Fig. 5. Separation of benzoylated polyamines from cultured PC-93 tumour cells. Cell pellets were treated with 0.3 *M* perchloric acid. (A) Untreated control cells; (B) cells grown for four days in the presence of 1 m*M* DFMO; (C) cells grown for four days in the presence of 1 m*M* CHAS. Elution with methanol–water 65:35 (v/v) for A and B; 60:40 (v/v) for C.

in quadruplicate, were: 104.7±3.4% for putrescine, 97.3±3.7% for spermidine and 88.8±5.5% for spermine.

The precision of the assay was tested by repeated analysis of (standard) mixtures of polyamines. From ten determinations on the same sample (containing ca. 10 nmol of each polyamine per 0.1 ml), performed within one analysis, the coefficient of variation (C.V.) was found to be 2.8% for putrescine, 4.4% for spermidine and 3.4% for spermine (intra-assay variation). When the same sample was analysed ten times in order to determine the day-to-day precision, the C.V. values were 8.1% for putrescine, 3.6% for spermidine and 3.4% for spermine (inter-assay variation). With PC-93 tumour cells homogenates, containing relatively low concentrations of putrescine (Table II), similar C.V. values were obtained (intra-assay variation: 2.9, 3.5 and 2.5%; inter-assay variation: 13.1, 8.2 and 9.6% for putrescine, spermidine and spermine, respectively).

DISCUSSION

The results presented above demonstrate that HPLC separation of benzoylated derivatives is a useful method for the analysis of di- and polyamines. The

procedure was originally described by Redmond and Tseng [15] and applied recently by others [16,25]. However, very few data on the characteristics of the assay (such as sensitivity, reproducibility and possible interferences) have been published so far. In the present work we have extensively characterized several important features of the assay after modification of the original procedure. A major improvement of the assay was achieved by the detection of the separated derivatives at 229 nm, the UV absorbance maximum of benzoyl groups (cf. Fig. 2), instead of 254 nm. Further modifications include the omission of high salt concentrations during the extraction step and the use of chloroform instead of diethyl ether as the extracting solvent. Chloroform was found to be superior to diethyl ether as it achieved a more complete extraction of the derivatives of all polyamines.

In contrast to diethyl ether, chloroform also extracted the benzoyl derivatives of monoacetylated polyamines. The observation that the peak-area ratios of acetylpolyamines were lower than those of the unconjugated compounds is explained by the fact that in the acetylated compounds there is one less amine group available for derivatization with benzoyl chloride. The extraction of toluoyl derivatives of acetylpolyamines (by diethyl ether), as reported earlier [25], should probably be ascribed to the different polarity of toluoylated and benzoylated polyamines. Acetylated polyamines do usually not accumulate in tumour tissues [1,2], although exceptions have been reported [26]. Since there was no evidence that significant amounts of acetyl-conjugated polyamines occurred in the cells and tissues studied by us, we did not investigate the analytical aspects further. Our results, however, indicate that in principle the assay might also be applied to the acetylated compounds (which are major components in extracellular fluids [27]), although the separation of the isomeric monoacetylspermidines might require the use of ternary solvent systems [28,29].

Although a variety of procedures for the determination of polyamine concentrations have been described already (for review, see ref. 9), the present method might be considered as a useful alternative, based on the detection by means of UV absorbance measurement (a highly versatile mode of detection that has been used in an increasing number of applications during the past several years). The sensitivity of the assay, modified as reported in this paper, is comparable with [10,13,14] or slightly below [12] that of other HPLC-based procedures, employing fluorescence detection after pre-chromatographic derivatization with dansyl chloride [10,12] or post-column derivatization with *o*-phthalaldehyde [13,14]. The use of isocratic conditions for the elution contributed to an excellent baseline stability and, thereby, to an optimal sensitivity which is sufficient to quantify polyamine levels in relatively small amounts of material (e.g. 10^5 tumour cells or less than 1 mg of tumour tissue), even after treatment with polyamine synthesis inhibitors.

A potential disadvantage of the use of UV absorbance detection, its lower selectivity, was found to be of little importance, due to the almost complete absence of interfering (UV-absorbing) substances, at least in the analysis of tumour cells and tissues. On the contrary, the high resolution offered by the HPLC technique

allowed for the quantitation of certain related compounds, such as the inhibitors CHAS and MAP, in addition to the polyamines in the same run.

The main advantage of the benzoylation method over most of the other published procedures is its simplicity. The method requires a minimum of sample pretreatment and employs a simple, but quantitative derivatization procedure. The time to perform a complete analysis (10 or 15 min) is much shorter than in most other assay methods and, in addition, re-equilibration between two samples is not necessary since isocratic elution is applied.

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REFERENCES

1 N. Seiler, F.N. Bolkenius and B. Knödgen, Biochim. Biophys. Acta, 633 (1980) 181.
2 N. Seiler, F.N. Bolkenius and S. Sarhan, Int. J. Biochem., 13 (1981) 1205.
3 J. Jänne, H. Pösö and A. Raina, Biochim. Biophys. Acta, 473 (1978) 241.
4 O. Heby, Differentiation, 14 (1981) 1.
5 M.H. Goyns, J. Theor. Biol., 97 (1982) 577.
6 A.E. Pegg and P.P. McCann, Am. J. Physiol., 243 (1982) C212.
7 J Koch-Weser, P.J. Schechter, P. Bey, C. Danzin, J.R. Fozard, M.J. Jung, P.S. Mamont, N. Seiler, N.J. Prakash and A. Sjoerdsma, in D.R. Morris and L.J. Marton (Editors), Polyamines in Biology and Medicine, Marcel Dekker, New York, 1981, Ch. 23, p. 437.
8 J. Jänne, E. Hölttä, A. Kallio and K. Käpyaho, in M.P. Cohen and P.P. Foa (Editors), Special Topics in Endocrinology and Metabolism, Vol. 5, Alan R. Liss, New York, 1983, p. 227.
9 N. Seiler, J. Chromatogr., 379 (1986) 157.
10 N. Seiler, B. Knödgen and F. Eisenbeiss, J. Chromatogr., 145 (1978) 29.
11 R.F. Minchin and G.R. Hanau, J. Liq. Chromatogr., 7 (1984) 2605.
12 P.M. Kabra, H.K. Lee, W.P Lubich and L.J. Marton, J. Chromatogr., 380 (1986) 19.
13 N. Seiler and B. Knödgen, J. Chromatogr., 221 (1980) 227.
14 W.L. Whitmore and T.A. Slotkin, Experientia, 41 (1985) 1209.
15 J.W. Redmond and A. Tseng, J. Chromatogr., 170 (1979) 479.
16 R. Porta, M. Camardella, V. Gentile and A. de Santis, J. Neurochem., 42 (1984) 321.
17 H. Hibasami, M. Tanaka, J. Nagai and T. Ikeda, FEBS Lett., 116 (1980) 99.
18 K.W Batchelor, R.A. Smith and N.S. Watson, Biochem J., 223 (1986) 307.
19 J.C. Romijn, C.F. Verkoelen and F.H. Schroeder, Cell Biol. Int. Rep., 8 (1984) 363.
20 J.C. Romijn, G.J. van Steenbrugge and F.H. Schroeder, in B. Sordat (Editor), Proceedings of the 4th International Workshop on Immune-Deficient Animals in Experimental Research, Chexbres, 1982, Karger, Basel, 1984, p. 370.
21 B.W. Metcalf, P. Bey, C. Danzin, M J. Jung, P. Casara and J.P. Vevert, J. Am. Chem. Soc., 100 (1978) 2551.
22 C Danzin, P. Casara, N. Claverie, B W. Metcalf and M.J. Jung, Biochem. Biophys. Res. Comm., 116 (1983) 237.
23 J C. Romijn, C.F. Verkoelen and T.A.W. Splinter, Cancer Chemother. Pharmacol., 19 (1987) 30.
24 F.A.J Muskiet, G.A. van den Berg, A.W. Kingma, D.C. Fremouw-Ottevangers and M.R Halie, Clin. Chem , 30 (1984) 687.
25 J.R. Clarke and A.S. Tyms, Med. Lab. Sci., 43 (1986) 258.
26 S. Takenoshita, S. Matsuzaki, G. Nakano, H. Kimura, H. Hoshi, H. Shoda and T. Nakamura, Cancer Res., 44 (1984) 845.

27 N. Seiler and B. Knödgen, J. Chromatogr., 164 (1979) 155.
28 N. Seiler and B. Knödgen, J. Chromatogr., 339 (1985) 45.
29 C. Stefanelli, D. Carati and C. Rossoni, J. Chromatogr., 375 (1986) 49.