Journal of Chromatography, 100 (1974) 95–100

CHROM. 7750

SEPARATION AND DETERMINATION OF NANOMOLE AMOUNTS OF SOME PURINES, URIC ACID AND URIC ACID RIBOSIDE BY TWO-DIMENSIONAL PAPER CHROMATOGRAPHY

MARIA KRZYŻANOWSKA and W. NIEMIERKO
Nencki Institute of Experimental Biology, Pasteur str. 3, 02-093 Warsaw (Poland)
(Received April 1st, 1974)

SUMMARY

A two-dimensional paper chromatographic method is described which allows good separations and spectrophotometric determinations of nanomole amounts of hypoxanthine, xanthine, uric acid and uric acid riboside. The method can be applied to biological material containing adenine nucleotides and considerable amounts of free amino acids.

INTRODUCTION

In an earlier paper[1], we described a simple procedure of continuous ascending paper chromatography which allowed the separation and quantitative determination of microgram amounts of adenine nucleotides and uric acid. When applying the method in our studies on insect biochemistry, we found that in some cases it was unsuitable. The wax moth larvae (*Galleria mellonella*), which were the chief object of our investigation, during starvation accumulate and excrete in addition to uric acid, which is the chief end product of nitrogen metabolism in most insects, also uric acid riboside[2,3], hypoxanthine and xanthine[4]. The simultaneous presence of these constituents made our earlier procedure inadequate, especially as the high concentration of free amino acids in the hemolymph, which is a characteristic feature of all insects[5], was also unfavourable.

After a number of preliminary experiments, we developed a two-dimensional paper chromatographic method which allowed an excellent separation of hypoxanthine (Hyp), xanthine (Xan), uric acid (UA) and uric acid riboside (UAR) and, furthermore, also adenine (Ade) and adenosine (Ado) which were added as possible splitting products of ATP. When these compounds were present in the mixture, each in an amount from several to about 100 nmole, a fairly good quantitative spectrophotometric determination could also be achieved. The addition to the mixture of adenine nucleotides and of a variety of amino acids was without any influence upon the separation of the investigated purines and uric acid riboside.

EXPERIMENTAL

All the compounds to be separated and all other chemicals and solvents were of laboratory reagent grade, purchased chiefly from Fluka (Buchs, Switzerland), Boehringer (Mannheim, G.F.R.) or BDH (Poole, Great Britain). Uric acid riboside (a synthetic ribosyl-3-uric acid preparation) was a gift from Prof. D. Shugar. Whatman No. 20 chromatography paper was used in all experiments: this type of paper appeared to give a sharper separation of the investigated compounds than the No. 1 paper which was used previously. The paper was washed with 0.4% EDTA solution and afterwards with distilled water; these washings were made by the continuous ascending technique described earlier[1]. Finally, the paper was dried in air at room temperature.

The investigated compounds were dissolved as individual solutions in 1% lithium carbonate in various concentrations from about 1 to about 10 m*M*. For each series of analyses, freshly prepared solutions were used.

The compounds to be analyzed were applied to the paper by placing samples of each of the individual solutions, one after the other, on the starting point at the lower left-hand corner of the same sheet of paper (18 × 28.5 cm) at a distance 3 cm from the edges. The volume of each solution applied was 7.4 μl, measured in each case with the "doubly constricted" micro-pipette shown in Fig. 1*.

After evaporation of the spotted samples in a gentle current of air, chromatographic development was performed in rectangular glass jars (20 × 12 × 20 cm high) covered with three tightly adjoining Teflon (DuPont, Wilmington, Del., U.S.A.) or Gaflon (Gachot, Enchien, France) plates. The paper strips (two in each jar) were firmly held between the plates by means of two fasteners. The first development was made with *n*-propanol–1% ammonia solution (60:40) for 17 h by the continuous ascending procedure described earlier[1]. The chromatograms were then removed from the vessels, dried in air for a few hours, the upper parts of the sheets (just below the "front") were cut out, the chromatograms were turned at right angles to the first run, suspended by means of cotton threads in further rectangular glass jars covered with glass plates and were developed by an ascending method with a solution of 5% disodium hydrogen orthophosphate saturated with isoamyl alcohol. This second development usually required 4 h: the solvent moved during this period through a height of about 15 cm. After drying in air, the detected spots were outlined under UV light with a soft pencil, the corresponding areas were cut out and eluted with 3.00 ml of 0.01% lithium carbonate solution by a procedure described previously[1]. The particular compounds were identified according to their positions on the chromatogram found in preliminary analyses and, additionally, according to their absorption curves. The quantitative spectrophotometric determinations were made in semi-micro quartz cells (2 cm path-length) using a Specord UV–VIS recording spectrophotometer (Carl Zeiss Jena, Jena, G.D.R.). The blanks consisted of eluates

* These simple micro-pipettes of our design were easily made by the glassblower in our laboratory in various sizes, from several to over 50 μl. Each of the pipettes was calibrated individually: the tolerances, even for the smallest sizes, were usually ± 1–2%. A special advantage of the pipettes is that the volumes of the solutions either measured directly into a glass vessel, the tip being in contact with the wall, or applied to the sheet of chromatographic paper, remain virtually constant. The liquid is drawn from the upper to the lower constriction by a very slight knocking.

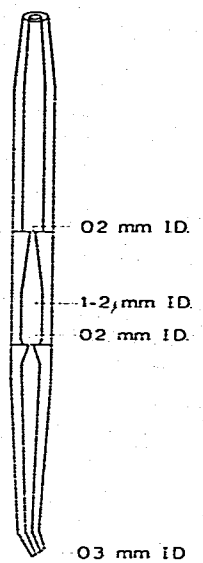

Fig. 1. A "doubly constricted" micropipette.

(0.01% lithium carbonate solution) prepared in an analogous manner from areas (of the same chromatograms) that did not contain UV-absorbing substances. For calculations, the appropriate specific absorptions were used.

RESULTS AND DISCUSSION

Six series of experiments were performed in which about 30 paper chromatograms were analyzed. Each of them contained mixtures of the complete range of various amounts of Ado, Ade, Hyp, Xan, UA and UAR which were separated by the two-dimensional technique as described above.

Because in all cases the results of the separations were virtually identical, it is sufficient to present as an example one typical chromatogram (taken from series No. 3) in which the separated compounds, as in all other cases, were rendered visible under UV light. This chromatogram is shown in Fig. 2.

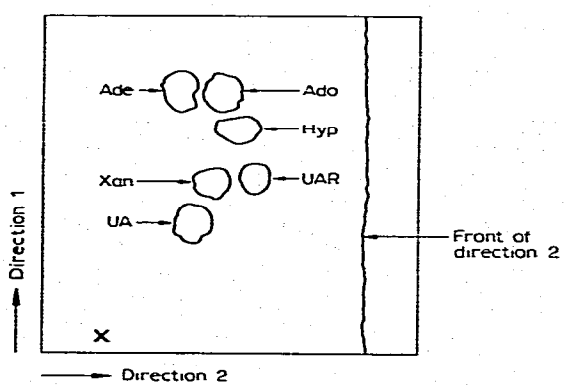

Fig. 2. Separation of Ade, Ado, Hyp, Xan, UA and UAR by two-dimensional paper chromatography.

TABLE I

QUANTITATIVE SPECTROPHOTOMETRIC DETERMINATION OF SOME PURINES AND NUCLEOSIDES AFTER SEPARATION BY TWO-DIMENSIONAL PAPER CHROMATOGRAPHY

Compound	*Series 1*		*Series 2*		*Series 3*		*Series 4*		*Series 5*		*Seiries 6*	
	Found directly (nmole)*	*Recovered** (nmole)*	*Found directly* (nmole)*	*Recovered** (nmole)*	*Found directly* (nmole)*	*Recovered** (nmole)*	*Found directly* (nmole)*	*Recovered** (nmole)*	*Found directly* (nmole)*	*Recovered** (nmole)*	*Found directly* (nmole)*	*Recovered** (nmole)*
Ade	7.5	6.3±1.5 (4)	7.7	6.2±0.2 (5)	20.0	16.6±0.3 (5)	37.0	28.5±1.0 (5)	74.0	54.5±0.7 (5)	148.0	98.0±3.5 (5)
Ado	4.7	3.0±0.4 (4)	9.5	7.6±0.2 (5)	28.8	24.1±1.2 (5)	23.5	21.3±1.0 (4)	92.0	71.8±1.0 (5)	184.0	152.4±5.1 (5)
Hyp	13.2	11.7±0.4 (5)	13.2	11.5±1.2 (4)	34.6	27.8±1.6 (6)	42.6	36.1±6.0 (4)	90.0	76.8±0.8 (5)	154.0	149.2±2.7 (4)
Xan	10.0	8.9±0.2 (5)	10.9	8.7±0.4 (5)	30.2	26.1±1.2 (5)	24.4	21.6±0.9 (5)	73.0	67.6±2.1 (5)	146.0	126.5±2.6 (5)
UA	8.1	6.7±0.3 (5)	9.2	6.0±0.3 (5)	28.0	23.1±0.7 (5)	43.0	34.4±0.8 (5)	82.4	74.8±5.2 (5)	164.8	134.8±4.0 (5)
UAR	5.0	3.3±0.1 (5)	7.8	5.9±0.4 (5)	22.3	17.3±0.4 (5)	26.4	19.7±0.3 (5)	54.1	51.4±1.8 (5)	108.2	97.8±2.5 (5)

* Amount found directly in the solutions of individual compounds (7.4-μl samples diluted to 3.00 ml with 0.01% lithium carbonate solution; mean values of two determinations).

** Amount recovered after two-dimensional separation of the mixture formed placing on the paper 7.4-μl samples of the solutions of each of the six investigated compounds; the corresponding spots (*cf.*, Fig. 2) were eluted with 3.00 ml of 0.01% lithium carbonate solution; the results are mean values ± S.E.M.; the number of experiments is shown in parentheses.

Table I summarizes all the results of the quantitative determinations of each of the investigated compounds eluted after the separation. It can be seen that although a small proportion of each compound appeared to be lost during the procedure, the mean recovery for the whole set of results was about 80%. The absolute values of S.E.M.* were as a rule very small, being often less than 1 nmole.

In preliminary analyses, we found in a number of cases that the amount of Hyp eluted from the chromatogram after the two-dimensional separation was up to 20% higher than that found directly in the samples of the solution of this compound (these cases are not included in Table I). We therefore assumed initially that some or even each of the remaining compounds might contain as an impurity small amounts of Hyp: such a possibility did not seem improbable. It appeared, however, that this was not true, because when all the compounds except Hyp were applied to the paper and developed in the usual manner, no traces of UV-absorbing substances were observed at the position corresponding to Hyp. We believe, therefore, that the apparent higher recovery of Hyp might be the result of a contamination of the cut-out spot of Hyp by peripheral parts of the spots of other compounds which are in the neighbourhood, most probably that of Ado. The practical conclusion of these observations is that in order to obtain reliable results, one must be very careful when cutting out individual spots from the chromatograms.

We then applied to the chromatographic paper Hyp, Xan, UA and UAR and also some small (nanomole) amounts of adenosine 5'-mono-(AMP), -di-(ADP) and -tri-(ATP) phosphate and five amino acids (Gly, Ala, Val, Met and Asp).

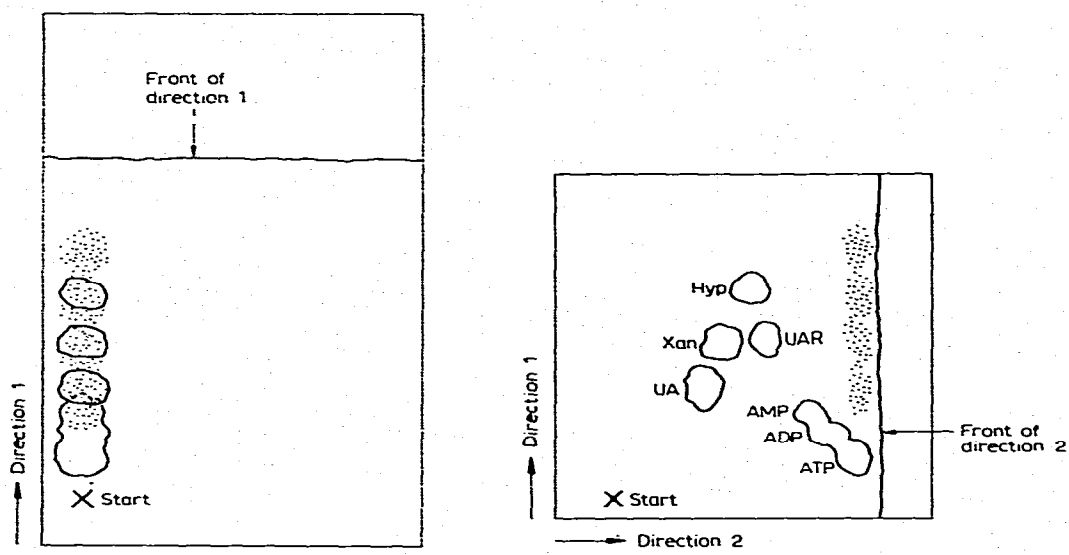

Fig. 3. Separation of Hyp, Xan, UA and UAR in the presence of AMP, ADP, ATP and a variety of amino acids after the first run by continuous ascending chromatography. The detected spots were outlined under UV light with a soft pencil and the amino acids subsequently rendered visible by spraying the chromatogram with a ninhydrin solution in acetone (they are shown as a series of dots).

Fig. 4. A parallel separation of the same substances as in Fig. 3 after two-dimensional paper chromatography.

* S. E. M. = standard error of the mean value.

Fig. 3 shows that after the first run with the solvent mixture *n*-propanol–1% ammonia solution (60:40) according to our continuous ascending method, the compounds overlap with each other. When, however, the next development was performed in the second direction with 5% disodium hydrogen orthophosphate saturated with isoamyl alcohol (this solvent mixture was found by Laster and Blair[6] to be particularly useful for separating UAR from UA), we found that all the amino acids moved just below the front and that the nucleotides had a high R_F value, although they moved close to each other. Fig. 4 shows clearly that also under these conditions the separation of Hyp, Xan, UA and UAR, which were the chief compounds of interest, was as sharp as previously (see Fig. 2).

REFERENCES

1 W. Niemierko and M. Krzyżanowska, *J. Chromatogr.*, 26 (1967) 424.
2 M. Krzyżanowska and W. Niemierko, *Bull. Acad. Polon. Sci., Sér. Sci. Biol.*, 18 (1970) 673.
3 M. Krzyżanowska and W. Niemierko, *X Congress Polish Biochem. Soc., Poznań, Abstr. Commun.*, 1972, p. 182 (in Polish).
4 M. Krzyżanowska and W. Niemierko, *XI Congress Polish Biochem. Soc., Białystok, Abstr. Commun.*, 1973, p. 196 (in Polish).
5 M. Florkin, in *Proceedings of the IV Int. Congress Biochem., Vienna, 1958* Pergamon Press, Elmsford, N.Y., 1959, Vol. 12, p. 63.
6 L. Laster and A. Blair, *J. Biol. Chem.*, 238 (1963) 3348.